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Characterization of Orf Y in the PurL Gene Cluster of Acetobacter aceti

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CHARACTERIZATION OF ORFY IN THE PURL GENE CLUSTER OF *ACETOBACTER ACETI*

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ABSTRACT

Chuong, Aurelie. MS, Purdue University, August 2015. Characterization of OrfY in the PurL Gene Cluster of *Acetobacter aceti*. Major Professor: T. Joseph Kappock.

FGAM synthetase, or PurL, catalyzes the fourth step of the *de novo* purine biosynthetic pathway where *N*-formylglycinamide ribonucleotide (FGAR), ATP, and glutamine yield *N*-formylglycinamidine ribonucleotide (FGAM), ADP, P_i, and glutamate. Two forms of PurL have been observed: one large, found in Gram-negative bacteria and eukaryotes; and the other, small, found in Gram-positive bacteria and archaea. Large PurL is a single gene product that is functional as a monomer whereas small PurL forms a $\alpha\beta_2\gamma$ heterotetramer with two other gene products: PurS and PurQ, which are analogous to the N-terminal and glutaminase domains of large PurL, respectively. Regardless of quaternary structure, all FGAM synthetases are thought to use ATP to *O*-phosphorylate the FGAR formamide, which is then attacked by ammonia to give FGAM.

The acetic acid bacterium *Acetobacter aceti*, and closely related α-proteobacteria, differ from other Gram-negatives in that they contain *purSQL* genes. The structural genes for the PurSQL complex (AaFS) are clustered into an apparent operon, which also contains an open reading frame (orf) that we designate *orf Y* and two genes (*bolA* and *grxD*) that have key roles in the assembly of iron-sulfur clusters. OrfY has a narrow species distribution: it is found in most acetic acid bacteria and several closely related Gram-negative bacteria. OrfY possesses four conserved cysteines and an N-terminal export sequence typical of periplasmic or exported proteins. Given this unusual set of features, and the likelihood of coupled transcription-translation in the *purSQYL-bolA-*

grxD operon, we speculate that OrfY might be an additional subunit for PurSQL, perhaps required for function in the acidic cytoplasm of acetic acid bacteria; a participant in iron-sulfur cluster assembly, which is essential for the production of the first purine pathway enzyme PurF and therefore purine biosynthesis; or as a regulatory factor. As a first step in understanding the role of OrfY, we sought to establish the functional form of the *A. aceti* enzyme to determine if *orf Y* encodes a heretofore unknown fourth subunit of the small PurL complex. This study focused on construct generation of various purine biosynthetic genes, purification of the proteins they encode, and preliminary work on gene complementation, the operon, and growth of *A. aceti* in minimal media.

Functional complementation studies in an *Escherichia coli* Δ*purL* strain have shown that active AaFS is likely composed of PurS, PurQ, and PurL, although the stoichiometry of the subunits is still unknown, thus indicating that OrfY is likely not a subunit of the AaFS complex. The presence of *orfY* in the gene cluster affected recombinant PurQ expression and caused a delay in growth in the *E.coli* Δ*purL* strain. Recombinant OrfY was successfully purified from *E. coli* and mass spectrometry analysis revealed that OrfY was processed and exported.

CHAPTER 1. CONSTRUCT GENERATION AND PURIFICATION OF AAFS SUBUNITS

1.1 Introduction

FGAM synthase (FS) catalyzes the fourth step of the *de novo* purine biosynthesis pathway, which makes purine bases from phosphoribosyl pyrophosphate (PRPP) through a series of enzyme-catalyzed steps [1]. FS converts formyglycinamide ribonucleotide (FGAR), ATP, and L-glutamine, to formylglycinamidine ribonucleotide (FGAM), ADP, Pi, and L-glutamate [1, 2]). Figure 1.1 shows a simplified de novo purine biosynthesis pathway, with an emphasis on the FS-catalyzed FGAR conversion to FGAM step.

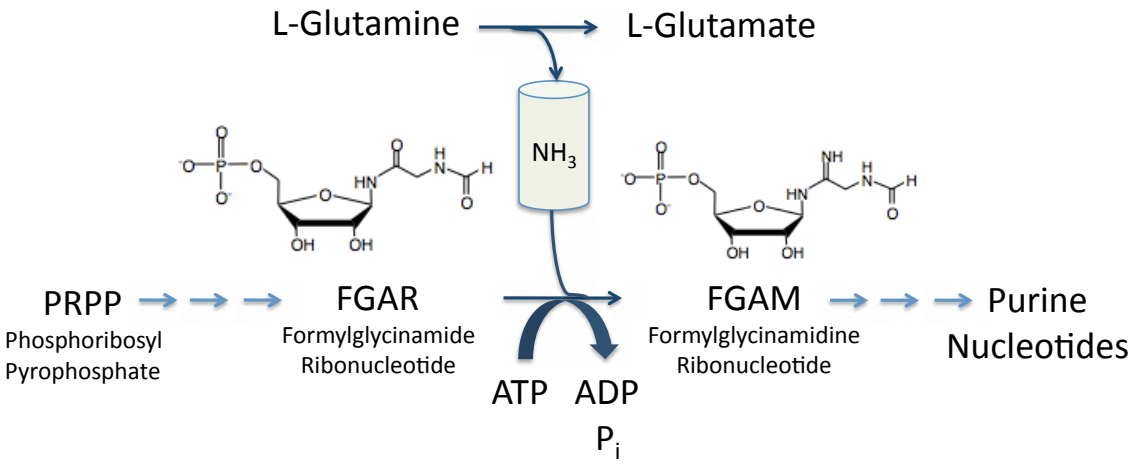

Figure 1.1 The FGAR to FGAM reaction catalyzed by FS.

The glutaminase domain hydrolyzes L-glutamine to L-glutamate and ammonia, the latter which travels to the FGAM synthetase domain via a channel linking the two active sites. Nucleophilic attack of ammonia onto the carbonyl carbon of the O-phosphorylated activated amide and deprotonation yields FGAM.

FS exists in two forms: the first, a 1300 amino acid-long polypeptide, or "large PurL", is found in Gram-negative bacteria (i.e. *Escherichia coli*, EcFS) and eukaryotes; and the second, "small PurL", of about 700 amino acids, is found in Gram-positive bacteria (i.e. *Bacillus subtilis*, BsFS) and archaea [1].

Multi-subunit FS studies have demonstrated that PurS and PurQ correspond to the N-terminal and glutaminase domains of large PurL, respectively [3-6]. The stoichiometric ratio of the BsFS components was determined to be 2 PurS : 1 PurQ : 1 PurL [7]. Crystal structures have also been solved for Gram-negative *Salmonella typhimurium* large PurL (StFS) and Gram-positive *Thermotoga maritima* small PurL (TmFS, Figure 1.2 [1]). Putative ammonia channels linking the glutaminase and FGAM synthetase active sites have been located in the StFS and TmFS crystal structures [5, 6]. This channel is critical to avoid protonation of ammonia to yield ammonium as demonstrated by the 50-fold activity increase seen with L-glutamine-dependent FGAM synthesis [7, 8]. Proper channeling of ammonia is likely a result of correct protein folding or FS complex assembly for maximum FS activity.

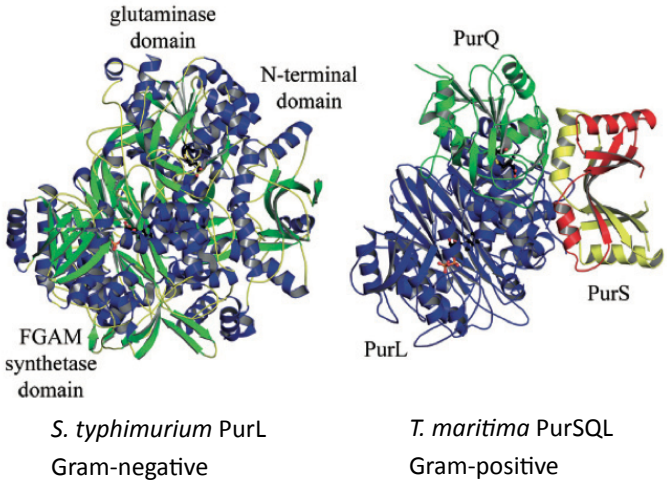

Figure 1.2 Crystal structures of large PurL and small PurL.

Large PurL of Gram-negative *S. typhimurium* (left) and small PurL of the PurSQL complex of Gram-positive *T. maritima* (right) [1]. The two forms of FS have visible structural similarities. PurQ corresponds to the glutaminase domain of large PurL; PurS is a dimer (right, in red and yellow) and corresponds to the N-terminal domain of large PurL. Ligands in the FGAM synthetase and PurL active sites is ADP; in the glutaminase and PurQ active sites is glutamine.

EcFS has been reported to require chaperone assistance for proper folding [9]; however, since the internal cavity of the well-studied chaperone GroEL-ES is apt to fit a ~70 kDa protein [10], many proteins require multiple rounds of folding [11]. A multi-subunit version of FS complex, composed of smaller and more easily folded proteins, may be an alternative and a selective advantage to make FS for organisms living in harsh environments (i.e. heat, salinity, acid). The dependence of small PurL on a chaperone system has not been reported. The Gram-negative *Acetobacter aceti*, best known for acetic acid production, is unusual due to its ability to survive with an acidic cytoplasm where the pH can range from 3.5 to 6 [12]. We hypothesize that acidophiles, like the Gram-negative *Acetobacter aceti*, have developed specialized mechanisms to prevent cytoplasmic acidification and/or conduct acid metabolism to keep their proteins folded and functional. Various cytoplasmic *A. aceti* proteins have been demonstrated to be acid-stable (and coincidentally thermo-stable) [13-16], where their stability is likely intrinsic to the primary structure of the proteins themselves. These proteins are thermodynamically and kinetically resistant to unfolding, which should lessen the burden on chaperones and on the cell.

AaFS is hypothesized to contain a multi-subunit FS, as suggested by gene cluster alignments with BsFS (Figure 1.3).

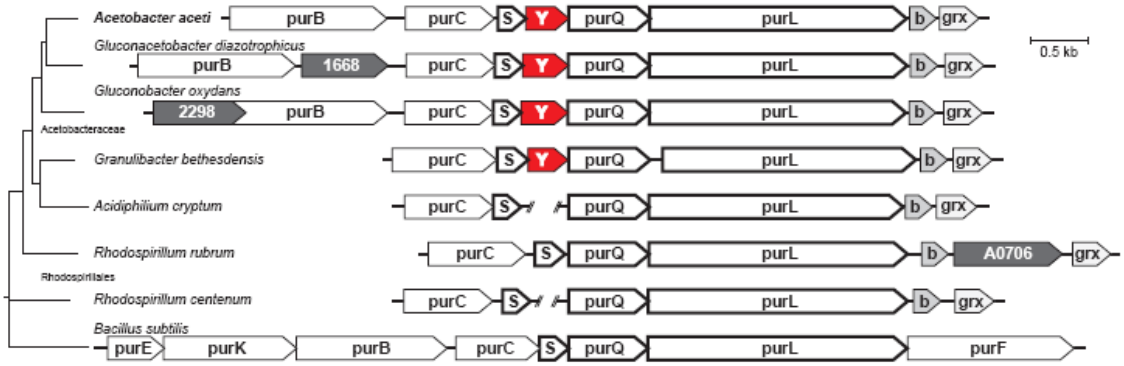

Figure 1.3 FS gene cluster alignment.

Includes select Gram-negative α-proteobacteria: *A. aceti*, Gram-negative *E. coli*, and Gram-positive *B. subtilis*. To date, acetic acid bacteria are the only Gram-negative bacteria that may contain PurS, PurQ, PurL, and even OrfY in some.
Legend: S = *purS* ; Y = *orfY*.

A multi-subunit AaFS is unexpected since *A. aceti* is Gram-negative; however closely related α-proteobacteria (Figure 3) also show the same gene cluster structure. Additionally, *A. aceti* and closely related acidophilic acetic acid bacteria also contain a previously uncharacterized open reading frame, *orfY*, located between *purS* and *purQ* (Figure 1.3).

This chapter focuses on construct design and purification of the tagged AaFS genes.

1.2 Materials and Methods

Materials

All materials were from Sigma Aldrich or Fisher Scientific and of the highest purity unless otherwise noted. Vent DNA polymerase, OneTaq polymerase, all restriction endonucleases, T4 DNA ligase, and T4 polynucleotide kinase were from New England Biolabs. Phusion Hot Start polymerase was from Finnzymes. GoTaq polymerase and deoxynucleotides (dATP, dGTP, dCTP, and dTTP) were from Promega. Mutagenesis was performed using QuikChange Mutagenesis kits from Stratagene (Pfu Polymerase). Oligodeoxynucleotides (ODNs) were from Integrated DNA Technologies (Table 1). Plasmid Miniprep kits were from either Sigma, Fermentas Life Sciences, or Qiagen. QIAquick PCR Purification kit was from Qiagen. Chemically competent DH5α cells were from Invitrogen. Vectors pET23a, pET23d, pET28a, pCDF-Duet, and pET-Duet were from Novagen. Centrifugation steps were performed on a tabletop Eppendorf 5415D centrifuge. PCR reactions were performed in a Bio-Rad MyCycler Thermal Cycler or a MJ Research Inc. Programmable Thermal Cycler PTC-100. *H6purS* and *H6orfY* in pUC57 (pJK518) were synthesized by GenScript. *A. aceti* 1023 was isolated using the Qiagen Genomic-tip 20/G (Elwood Mullins).

Table 1.1 Oligodeoxynucleotides used in construction of plasmids and sequencing.

ODN	Sequence (5'=>3')[a]
2074	GTGGTGCTCGAGTGAGTTGCCCATCAGCATCGGGA
2076	AGTGTTCCGACCACATCACA
2091	TAAGAAGGAGATATACATATGAAGGTACGTGTAACC
2092	GTGGTGGTGGTGGTGCTCGAGTTAGTTGCCCATCAGCATCGGGA
2103	TTCCCCGGTACCAACCGGGAGCGCGATATGG
2104	ATTGAAAAGCTTCTGATTGAAGCCTGTCTGGA
2105	CAGGCTTCAATCAGAAGCTTTTCAATAAAGGG
2113	GATATACATATGAAGCGCACCCTTCTTATAGCGCTGG
2115	CCGCGGCCATGGCGAAGGTACGTGTAACCGTCATGCTGAAG
2116	AAGGGTAAGCTTCATTCCACCACCTCTACAGAGTAATCTTCAATCACCAGGTTGGC TAGCAGATCACGCGCCATAGCATCGGCCTTTTTC
2117	GTGGTGCCATGGCAAAGCGCACCCTTCTTATAGCGCTGG
2118	TAATTGAAGCTTTCATTTTTTCACACTGCACGGATAAGAGG
2119	GGCAGCCATATGCAGCGCATTGCGCCCATGAAGG
2120	GTGGTGCTCGAGTGATTTTTTCACACTGCACGGATAAGAGG
2121	TGTGAACATATGAAAGCAGCAATTATCGTATTC
2122	TTACAGCTCGAGTCACCGGACTACAGCCTCC
2123	TGTAGTCATATGAATAAGCCTGTAACCGTTGATGAATC
2131	ATATACATATGAAAGCTGCGATTATAGTGTTTCCTGGTACTAACCGTGAGCGCGAT ATGG
2132	TATGAATAAGCCTGTAACCGTTGATGAATCCCTTGCCCGTG
2133	AATTCACGGGCAAGGGATTCATCAACGGTTACAGGCTTATTCA
2193	CTAGCCAACCTGGTGATTGAAGATTACTCTGTAGAGGTGGTGGAATGAAGCGCAC CCTTCTTATAGCGCTGGCATTGGCATG
2194	CCAATGCCAGCGCTATAAGAAGGGTGCGCTTCATTCCACCACCTCTACAGAGTAAT CTTCAATCACCAGGTTGG
2195	CTAGCCAACCTGGTGATTGAAGATTACTCTGTAGAGGTGGTGGAATGAAAGCAGC AATTATCGTATTCCCCGGTAC
2196	CGGGGAATACGATAATTGCTGCTTTCATTCCACCACCTCTACAGAGTAATCTTCAA TCACCAGGTTGG
2247	CGGGGAATACGATAATTGCTGCTTTCA
2248	TATGAAAGCAGCAATTATCGTATTCCCCGGTAC
2249	GATCCATGAAAGCAGCAATTATCGTATTCCCCGGTAC
2250	CGGGGAATACGATAATTGCTGCTTTCATG
2253	TTGCGGGAAGATTTGTAGGAGCAGTGTTCC
2254	GCCTGTGGGATATGCACACGCACGAAAAAC
2255	GGCAAAAACACTTTCACCAACCGGCTTGTC
2295	CACGCGTTTTCAcCGTTGGCATTGAG
2296	CTCAATGCCAACGgTGAAAACGCGTG
2332	AGTTCCCATATGTCCGCTGAACAC
2333	CTGCTTCTCGAGTTAGACGCTGTAGTA
2334	ATAGTGCTCGAGTCATCCTTCGTTATGC

[a]Letters shown in lower case code for mutagenesis.

Methods

General cloning procedure. Each recombinant gene was amplified by PCR with gene-specific primers (Table 1) *Acetobacter aceti* 1023 genomic DNA or plasmid DNA as specified below. *E. coli* strain DH5α was used for cloning and plasmid maintenance. All constructs were screened by restriction mapping or colony PCR (Chapter 6). All numbered plasmids were verified by DNA sequencing at the Genomics Core Facility DNA Sequencing Low Throughput Laboratory at Purdue University, using primers for both directions.

Table 1.2 Plasmids used in this chapter[a].

pJK	Vector	Description	Source
518	pUC57	*purS-H6orfY*	GenScript
520	pUC57	*purS-H6orfY*	TJK
522	pET28a	*H6purS-H6orfY*	TJK
529	pET23a	PCR B Mutant R430L (*purL* 2nd half)	This study
530	pET23d	*orfYH6*	This study
531	pET23d	*orfY*	This study
532	pET28a	*H6purQ* (silA63)	This study
533	pET23d	*purS*	This study
534	pET23a	*purQ* (Q10C and R13L)-PCR A_2	This study
535	pET23a	*purQ* (silA63)-*pur*L(G209D)	This study
537	pET23a	*purL*(G209D)	This study
539	pET23a	*purL*	This study
543	pCDF-Duet	*purS* and *purL*	This study
544	pCDF-Duet	*purS*	This study
546	pET-Duet	*orfY*	This study
548	pET-Duet	*orfY* and *purQ*	This study
549	pCDF-Duet	*purL*	This study
555	pET23a	*purQ*	This study
567	pCDF-Duet	*orfY*	This study
571	pUC57	*purS-H6orfY-purQ-purL*(G209D)	This study
574	pUC57	*purS-H6orfY-purQ-purL*	This study
575	pET23a	*purLH6*	This study
576	pUC57	*purS-orfY-purQ*(part)	This study
577	pUC57	*purS-purQ*(part)	This study
595	pUC57	*purS-orfY-purQ-purL*(part/G209D)	This study
596	pUC57	*purS-purQ-purL*(part/G209D)	This study
598	pET23a	*purS-orfY-purQ-purL*	This study

[a]Hyphens indicate overlapping genes (as in the native *A. aceti* sequence).

Table 1.2 (continued) Plasmids used in this chapter[a].

599	pET23a	*purS-orfY-purQ-purLH6*	This study
605	pET23a	*purS-purQ-purL*	This study
606	pET23a	*purS-purQ-purLH6*	This study
610	pET23a	*purQ-purL*	This study
611	pET23a	*purQ-purLH6*	This study
612	pET23a	*purS-orfYΔS70-K123-purQ-purL*	This study
613	pET23a	*purS-orfYΔS70-K123-purQ-purLH6*	This study
618	pET28a	*H6purS-purQ-purLH6*	This study
619	pET28a	*H6purS-orfY-purQ-purL*	This study
620	pET28a	*H6purS*	This study
622	pET28a	*H6purL*	This study
623	pET23a	*orfY-purQ-purL*	This study
624	pET23a	*orfY-purQ-purLH6*	This study
625	pET28a	*H6purS-purQ-purLH6*	This study

[a]Hyphens indicate overlapping genes (as in the native *A. aceti* sequence).

Synthetic *purS-H6orfY* construct plasmid pJK518 (TJK). Synthetic *purS* (silent D15 and L66 mutations), *H6orfY* (silent T40, G67, and H114 mutations) , and the first 45 bases of purQ were synthesized by GenScript. The plasmid encodes for PurS, H6OrfY, and the first 11 amino acids of PurQ.

Synthetic *purS-H6orfY* construct plasmid pJK520 (TJK). pJK518 was digested with NdeI to remove a 218 bp fragment and ligated to yield from pJK520. The plasmid encodes for PurS, H6OrfY, and the first 11 amino acids of PurQ.

Synthetic *H6purS-H6orfY* construct plasmid pJK522 (TJK). pJK520 was digested with NdeI and HindIII and ligated into the NdeI and HindIII restriction sites of the destination vector pET28a to yield pJK522 . The plasmid encodes for H6PurS, H6OrfY, and the first 11 amino acids of PurQ.

a. Cloning of *orfY*

Construction of single *orfYH6* construct plasmid pJK530. A 0.4 kb product containing *orfY* was amplified from template pJK522, using ODNs 2117 and 2120 with an annealing temperature of 60°C and melting, annealing, and extension times of 20 s, 30 s, and 30 s for 25 cycles using Vent DNA Polymerase. The product was purified using a Qiagen

PCR purification kit, digested with NcoI and HindIII, and ligated into the NcoI and HindIII sites of the destination vector pET23d to yield pJK530. The plasmid encodes for OrfYH6.

Construction of single *orfY* construct plasmid pJK531. A 0.4 kb product containing *orfY* was amplified from template pJK522, using ODNs 2117 and 2118 with an annealing temperature of 60°C and melting, annealing, and extension times of 20 s, 30 s, and 30 s for 25 cycles using Vent DNA Polymerase. The product was purified using a Qiagen PCR purification kit, digested with NcoI and HindIII, and ligated into the NcoI and HindIII sites of the destination vector pET23d to yield pJK530. The plasmid encodes for OrfY.

Construction of single *orfY* construct plasmid pJK567. pJK531 was digested with NcoI and HindIII and ligated into the NcoI and HindIII restriction sites of the destination vector pCDF-Duet to yield pJK567. The plasmid encodes for OrfY.

b. Cloning of *purS*

Construction of single *purS* construct plasmid pJK533. A 0.2 kb product containing *purS* was amplified from template pJK522, using ODNs 2115 and 2116 with an annealing temperature of 60°C and melting, annealing, and extension times of 20 s, 30 s, and 30 s for 25 cycles using Vent DNA Polymerase. The product was purified using a Qiagen PCR purification kit, digested with NcoI and HindIII, and ligated into the NcoI and HindIII sites of the destination vector pET23d to yield pJK533. The plasmid encodes for PurS.

Construction of single *H6purS* construct plasmid pJK620. A 0.2 kb product containing *purS* was amplified from template pJK533, using ODNs 2091 and 2116 with an annealing temperature of 55°C and melting, annealing, and extension times of 20 s, 30 s, and 30 s for 25 cycles using OneTaq Polymerase. The product was purified using a Qiagen PCR purification kit, digested with NdeI and HindIII, and ligated into the NdeI and HindIII sites of the destination vector pET28a to yield pJK620. The plasmid encodes for H6PurS.

c. Cloning of *purL*

Construction of intermediate construct plasmid pJK529. A 1.5 kb product containing the C-terminus of *purL* was amplified from *A. aceti* strain 1023 genomic DNA, using ODNs 2104 and 2092 (Figure 4) with an annealing temperature of 60°C and melting, annealing, and extension times of 20 s, 30 s, and 1 min 30 s for 25 cycles using Vent DNA Polymerase. The product was purified using a Qiagen PCR purification kit, digested with HindIII and XhoI, and ligated into the HindIII and XhoI restriction sites of the destination vector pET23a to yield pJK529. The plasmid encodes for the last 488 amino acids of PurL with a mutation R430L.

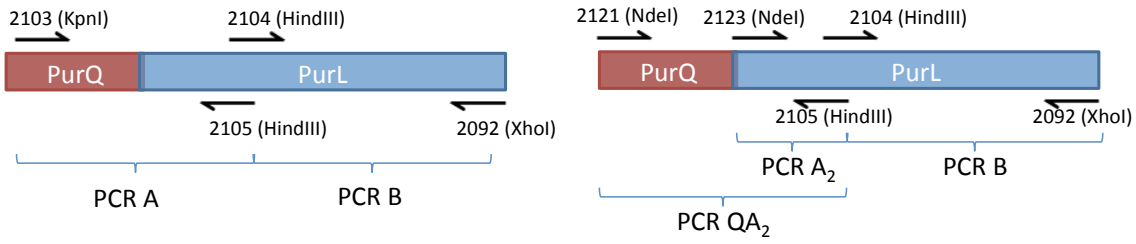

Figure 1.4 Schematic for building pJK529, pJK534, and pJK535.
Left, PCRs A and B; and right, PCRs QA_2, A_2, and B, relative to *purQ* and *purL* (genes not to scale) with ODNs used to obtain the PCR products.

The cloning strategy used to build the *purL* construct is summarized in Figure 5, on the next page.

Construction of intermediate construct plasmid pJK534. A 1.4 kb product containing *purQ* and the N-terminus of *purL* (PCR A in Figure 4) was amplified from *A. aceti* strain 1023 genomic DNA, using ODNs 2121 and 2105 with an annealing temperature of 60°C and melting, annealing, and extension times of 20 s, 30 s, and 1 min 30 s for 25 cycles using Vent DNA Polymerase. PCR A was purified using a Qiagen PCR purification kit, digested with NdeI and HindIII, and ligated into the NdeI and HindIII restriction sites of the destination vector pET23a to yield pJK534. The plasmid encodes for PurQ G10C and R13L (random mutations) and the first 247 amino acids of PurL.

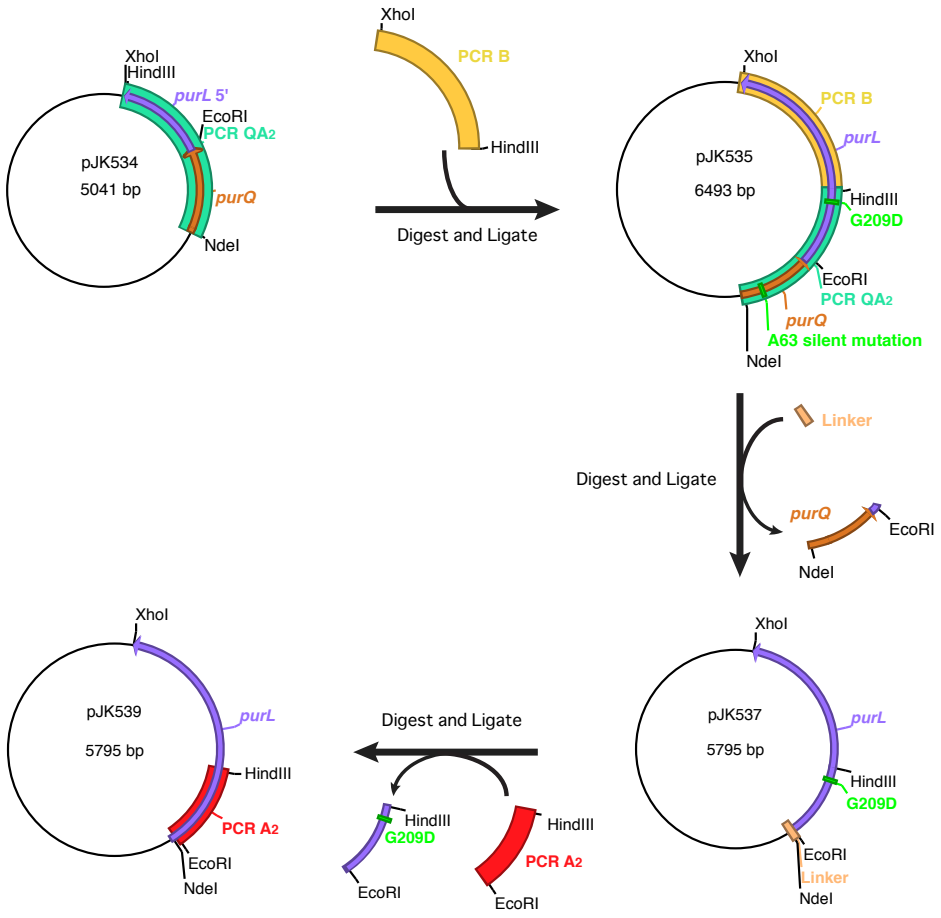

Figure 1.5 Summary of the construction of pJK539.

PCR B (yellow) was ligated into plasmid pJK534, which contains PCR QA_2 (green) , to yield plasmid pJK535. A linker (ODNs 2132 and 2133, tan) was then ligated into plasmid pJK535 after the excision of *purQ* (orange), to yield plasmid pJK537. PCR A_2 (red) was ligated into plasmid pJK537 to repair the G209D mutation in *purL* (purple). Plasmids not to scale.

Construction of intermediate construct plasmid pJK535. A 1.5 kb product containing the C-terminus of *purL* (PCR B in Figure 4) was amplified from *A. aceti* strain 1023 genomic DNA, using ODNs 2104 and 2092 with an annealing temperature of 60°C and melting, annealing, and extension times of 20 s, 30 s, and 1 min 30 s for 25 cycles using Vent DNA Polymerase. PCR B was purified using a Qiagen PCR purification kit, digested with HindIII and XhoI, and ligated into the HindIII and XhoI restriction sites of the destination vector pJK534. The plasmid encodes for PurQ silent A63 mutation (random mutation) and PurL G209D (random mutation).

Construction of intermediate construct plasmid pJK537. ODNs 2132 and 2133 were phosphorylated, annealed, and ligated at a 1:10 molar ratio of vector : linker into the NdeI and EcoRI sites of destination vector pJK537. The plasmid encodes for PurL G209D.

Construction of single *purL* construct plasmid pJK539. A 0.7 kb product (PCR A_2) containing the N-terminus of *purL* was amplified from *A. aceti* strain 1023 genomic DNA, using ODNs 2123 and 2105 with an annealing temperature of 60°C and melting, annealing, and extension times of 20 s, 30 s, and 1 min 30 s for 25 cycles using Vent DNA Polymerase. PCR A_2 was purified using a Qiagen PCR purification kit, digested with EcoRI and HindIII, and ligated into the EcoRI and HindIII restriction sites of the destination vector pJK537. The plasmid encodes for PurL.

Construction of single *purL* construct plasmid pJK549. pJK539 was digested with NdeI and XhoI and ligated into the NdeI and XhoI restriction sites of the destination vector pCDF-Duet to yield pJK549. The plasmid encodes for PurL.

Construction of single *purL* construct plasmid pJK575. A 2.2 kb product was amplified from template pJK539, using ODNs 2104 and 2074 with an annealing temperature of 60°C and melting, annealing, and extension times of 20 s, 30 s, and 2 min 30 s for 25 cycles using Vent DNA Polymerase. The product was purified using a Qiagen PCR purification kit, digested with NdeI and XhoI, and ligated into the NdeI and XhoI restriction sites of the destination vector pET23a. The plasmid encodes for PurLH6.

Construction of single *H6purL* construct plasmid pJK622. pJK539 was digested with NdeI and XhoI and ligated into the NdeI and XhoI restriction sites of the destination vector pET28a to yield pJK622. The plasmid encodes for H6PurL.

d. Cloning of purQ

Construction of single *H6purQ* construct plasmid pJK532. pJK535 was digested with NdeI and EcoRI and ligated into the NdeI and EcoRI restriction sites of the destination vector pET28a to yield pJK532. The plasmid encodes for H6PurQ.

Construction of single *purQ* construct plasmid pJK555. pJK532 was digested with NdeI and XhoI and ligated into the NdeI and XhoI restriction sites of the destination vector pET23a to yield pJK555. The plasmid encodes for PurQ.

e. Construction of double gene constructs

Construction of single *purS* construct plasmid pJK544. pJK533 was digested with NcoI and HindIII and ligated into the NcoI and HindIII restriction sites of the destination vector pCDF-Duet to yield pJK544. The plasmid encodes for PurS (with an additional Ala2 residue to accomodate the NcoI site).

Construction of double *purS* and *purL* construct plasmid pJK543. pJK539 was digested with NdeI and XhoI and ligated into the NdeI and XhoI restriction sites of the destination vector pJK544 to yield pJK543. The plasmid encodes for PurS (with an additional Ala2 residue to accomodate the NcoI site) and PurL.

Construction of single *orfY* construct plasmid pJK546. pJK531 was digested with NcoI and HindIII and ligated into the NcoI and HindIII restriction sites of the destination vector pET-Duet to yield pJK546. The plasmid encodes for OrfY (with an additional Ala2 residue to accomodate the NcoI site.

Construction of double *orfY* and *purQ* construct plasmid pJK548. pJK532 was digested with NdeI and XhoI and ligated into the NdeI and XhoI restriction sites of the destination vector pJK546 to yield pJK548. The plasmid encodes for OrfY((with an additional Ala2 residue to accomodate the NcoI site) and PurQ.

f. Cloning the overlapping 2-gene, 3-gene, and 4-gene constructs

The cloning strategies to build the AaFS 4-gene and 3-gene constructs are summarized in Figure 6 and 7, respectively.

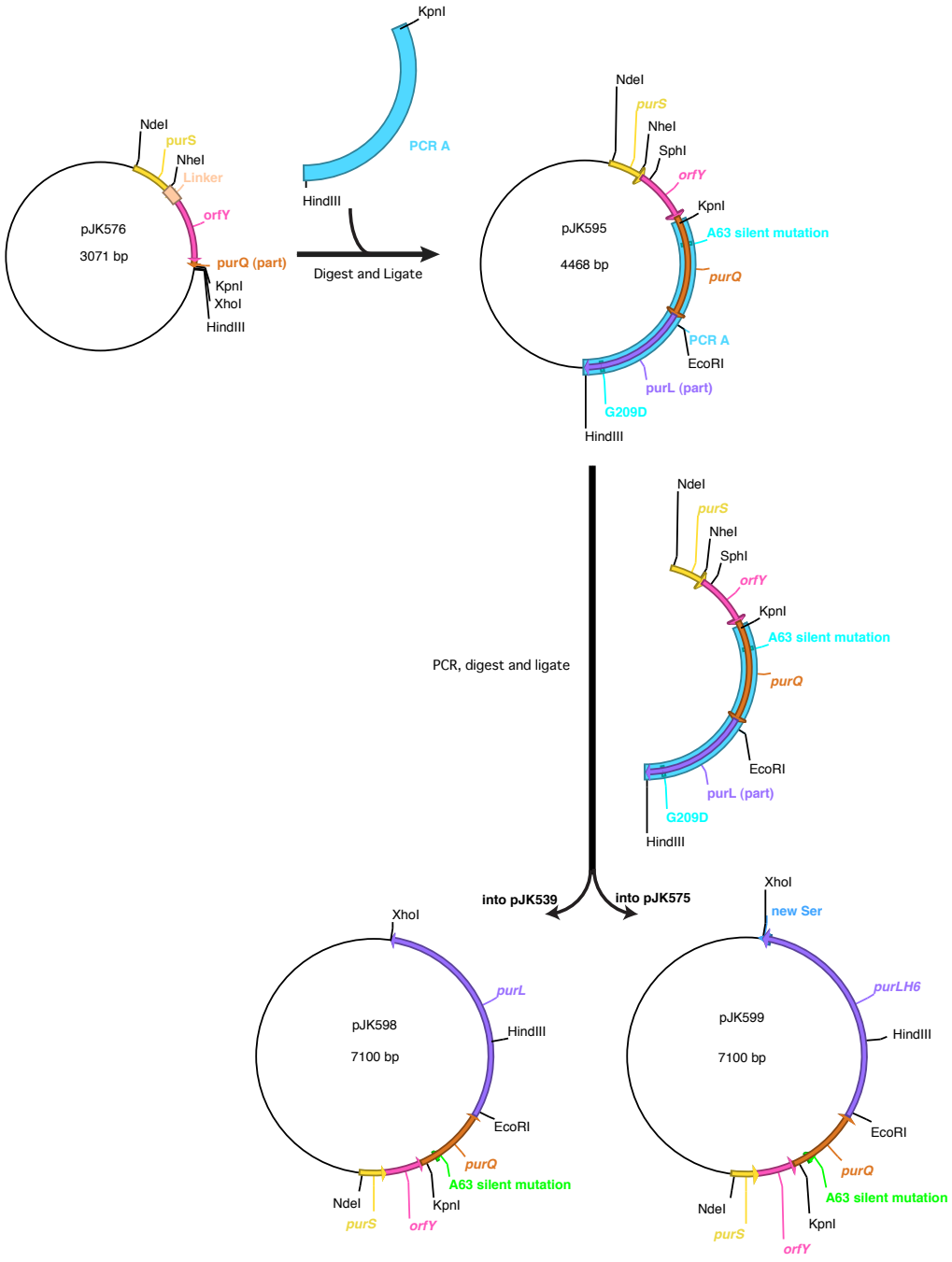

Figure 1.6 Summary of the construction of the AaFS 4-gene cluster.

PCR A (blue) was ligated into plasmid pJK576, which contains *purS* (yellow), *orfY* (pink), and part of *purQ* (orange), to yield plasmid pJK595. A PCR product spanning from the restriction sites NdeI through HindIII was obtained using ODNs 2091 and 2105 from template pJK595. This PCR product was ligated into plasmid pJK539 to complete the *purL* gene (purple) to yield plasmid pJK598. The same PCR product was also ligated into pJK575 to complete the *purLH6* sequence to yield plasmid 599. Plasmids are not to scale.

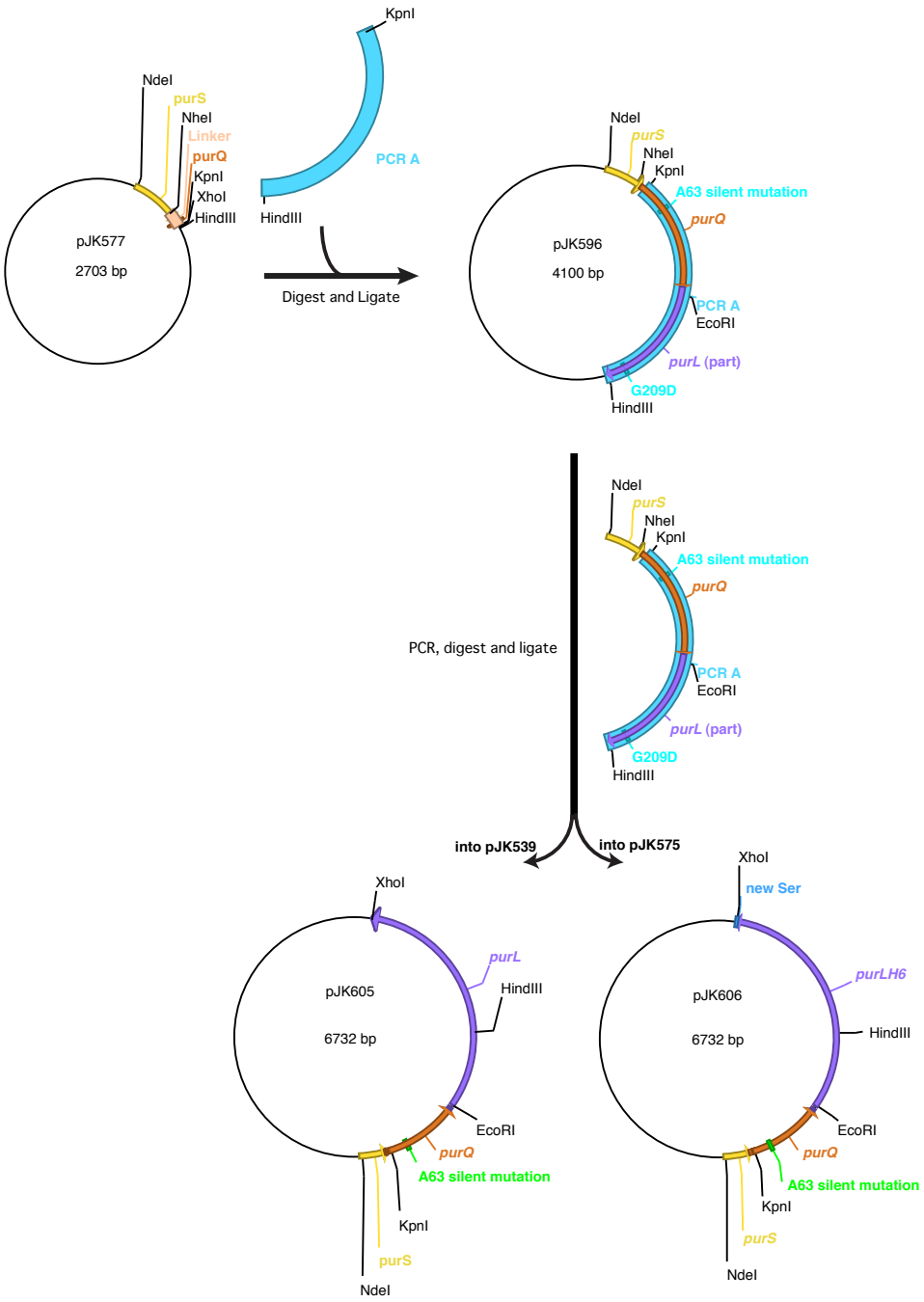

Figure 1.7 Summary of the construction of the AaFS 3-gene cluster.

PCR A (blue) was ligated into plasmid pJK577, which contains *purS* (yellow), and part of *purQ* (orange), to yield plasmid pJK596. A PCR product spanning from the restriction sites NdeI through HindIII was obtained using ODNs 2091 and 2105 from template pJK596. This PCR product was ligated into plasmid pJK539 to complete the *purL* gene (purple) to yield plasmid pJK605. The same PCR product was also ligated into pJK575 to complete the *purLH6* sequence to yield plasmid 606. Plasmids are not to scale.

Construction of intermediate *purS-H6orfY-purQ-purL* construct plasmid pJK571. A 2.9 kb product was amplified from template pJK535, using ODNs 2103 and 2092 with an annealing temperature of 60°C and melting, annealing, and extension times of 20 s, 30 s, and 2 min 30 s for 25 cycles using Vent DNA Polymerase. The product was purified using a Qiagen PCR purification kit, digested with KpnI and XhoI, and ligated into the KpnI and XhoI restriction sites of the destination vector pJK520 to yield pJK571. The plasmid encodes for PurS, H6OrfY, PurQ, and PurL G209D.

Construction of intermediate *purS*-H6o*rfY-purQ-purL* construct plasmid pJK574. pJK539 was digested with EcoRI and XhoI and ligated into the EcoRI and XhoI restriction sites of the destination vector pJK571 to yield pJK574. The plasmid encodes for PurS, H6OrfY, PurQ, and PurL.

Construction of intermediate *purS-orfY-purQ* construct plasmid pJK576. ODNs 2193 and 2194 were phosphorylated, annealed, and ligated at a 1:10 molar ratio of vector : linker into the NheI and SphI sites of destination vector pJK520 to yield pJK576. The plasmid encodes for PurS, OrfY, and the first 11 amino acids of PurQ.

Construction of intermediate *purS-purQ* construct plasmid pJK577. ODNs 2195 and 2196 were phosphorylated, annealed, and ligated at a 1:10 molar ratio of vector : linker into the NheI and SphI sites of destination vector pJK520 to yield pJK577. The plasmid encodes for PurS and the first 11 amino acids of PurQ.

Construction of *purS-orfY-purQ-purL* construct plasmid pJK595. A 1.5 kb product was amplified from template pJK535, using ODNs 2103 and 2105 with an annealing temperature of 60°C and melting, annealing, and extension times of 20 s, 30 s, and 1 min 30 s for 25 cycles using Vent DNA Polymerase. The product was purified using a Qiagen PCR purification kit, digested with KpnI and XhoI, and ligated into the KpnI and XhoI restriction sites of the destination vector pJK576 to yield pJK595. The plasmid encodes for PurS, OrfY, PurQ, and the first 247 amino acids of PurL G209D.

Construction of *purS-purQ-purL* construct plasmid pJK596. A 1.5 kb product was amplified from template pJK535, using ODNs 2103 and 2105 with an annealing temperature of 60°C and melting, annealing, and extension times of 20 s, 30 s, and 1 min 30 s for 25 cycles using Vent DNA Polymerase. The product was purified using a Qiagen PCR purification kit, digested with KpnI and XhoI, and ligated into the KpnI and XhoI restriction sites of the destination vector pJK577 to yield pJK596. The plasmid encodes for PurS, PurQ, and the first 247 amino acids of PurL G209D.

Construction of *purS-orfY-purQ-purL* construct plasmid pJK598. A 2.0 kb product was amplified from template pJK595, using ODNs 2091 and 2105 with an annealing temperature of 55°C and melting, annealing, and extension times of 20 s, 30 s, and 1 min 30 s for 25 cycles using OneTaq polymerase. The product was purified using a Qiagen PCR purification kit, digested with NdeI and EcoRI, and ligated into the NdeI and EcoRI restriction sites of the destination vector pJK539 to yield pJK598. The plasmid encodes for PurS, OrfY, PurQ, and PurL.

Construction of *purS-orfY-purQ-purLH6* construct plasmid pJK599. A 2.0 kb product was amplified from template pJK595, using ODNs 2091 and 2105 with an annealing temperature of 55°C and melting, annealing, and extension times of 20 s, 30 s, and 1 min 30 s for 25 cycles using OneTaq polymerase. The product was purified using a Qiagen PCR purification kit, digested with NdeI and EcoRI, and ligated into the NdeI and EcoRI restriction sites of the destination vector pJK575 to yield pJK599. The plasmid encodes for PurS, OrfY, PurQ, and PurLH6.

Construction of *purS-purQ-purL* construct plasmid pJK605. A 1.7 kb product was amplified from template pJK596, using ODNs 2091 and 2105 with an annealing temperature of 55°C and melting, annealing, and extension times of 20 s, 30 s, and 1 min 30 s for 25 cycles using OneTaq polymerase. The product was purified using a Qiagen PCR purification kit, digested with NdeI and EcoRI, and ligated into the NdeI and EcoRI restriction sites of the destination vector pJK539 to yield pJK605. The plasmid encodes for PurS, PurQ, and PurL.

Construction of *purS-purQ-purLH6* construct plasmid pJK606. A 1.7 kb product was amplified from template pJK596, using ODNs 2091 and 2105 with an annealing temperature of 55°C and melting, annealing, and extension times of 20 s, 30 s, and 1 min 30 s for 25 cycles using OneTaq polymerase. The product was purified using a Qiagen PCR purification kit, digested with NdeI and EcoRI, and ligated into the NdeI and EcoRI restriction sites of the destination vector pJK575 to yield pJK606. The plasmid encodes for PurS, PurQ, and PurLH6.

Construction of *purQ-purL* construct plasmid pJK610. ODNs 2247 and 2248 were phosphorylated, annealed, and ligated at a 1:10 molar ratio of vector : linker into the NdeI and KpnI sites of destination vector pJK605. The plasmid encodes for PurQ and PurL.

Construction of *purQ-purLH6* construct plasmid pJK611. ODNs 2247 and 2248 were phosphorylated, annealed, and ligated at a 1:10 molar ratio of vector : linker into the NdeI and KpnI sites of destination vector pJK606. The plasmid encodes for PurQ and PurLH6.

Construction of *orfY-purQ-purL* construct plasmid pJK612. ODNs 2249 and 2250 were phosphorylated, annealed, and ligated at a 1:10 molar ratio of vector : linker into the BamHI and KpnI sites of destination vector pJK598. The plasmid encodes for OrfY(ΔS70-K123), PurQ, and PurL.

Construction of *orfY-purQ-purLH6* construct plasmid pJK613. ODNs 2249 and 2250 were phosphorylated, annealed, and ligated at a 1:10 molar ratio of vector : linker into the BamHI and KpnI sites of destination vector pJK599. The plasmid encodes for OrfY(ΔS70-K123), PurQ, and PurLH6.

Construction of *H6purS-orfY-purQ-purLH6* construct plasmid pJK618. pJK599 was digested with NdeI and XhoI and ligated into the NdeI and XhoI restriction sites of the destination vector pET28a to yield pJK618. The plasmid encodes for H6PurS, OrfY, PurQ, and PurLH6.

Construction of *H6purS-orfY-purQ-purL* construct plasmid pJK619. pJK598 was digested with NdeI and XhoI and ligated into the NdeI and XhoI restriction sites of the destination vector pET28a to yield pJK619. The plasmid encodes for H6PurS, OrfY, PurQ, and PurL.

Construction of *orfY-purQ-purL* construct plasmid pJK623. A 1.3 kb product was amplified from template pJK598, using ODNs 2113 and 2076 with an annealing temperature of 55°C and melting, annealing, and extension times of 20 s, 30 s, and 30 s for 25 cycles using Phusion polymerase. The product was purified using a Qiagen PCR purification kit, digested with NdeI and EcoRI, and ligated into the NdeI and EcoRI restriction sites of the destination vector pJK539 to yield pJK623. The plasmid encodes for OrfY, PurQ, and PurL.

Construction of *orfY-purQ-purLH6* construct plasmid pJK623. A 1.3 kb product was amplified from template pJK598, using ODNs 2113 and 2076 with an annealing temperature of 55°C and melting, annealing, and extension times of 20 s, 30 s, and 30 s for 25 cycles using Phusion polymerase. The product was purified using a Qiagen PCR purification kit, digested with NdeI and EcoRI, and ligated into the NdeI and EcoRI restriction sites of the destination vector pJK575 to yield pJK624. The plasmid encodes for OrfY, PurQ, and PurLH6.

Construction of *H6purS-purQ-purL* construct plasmid pJK625. A 1.2 kb product was amplified from template pJK605, using ODNs 2091 and 2076 with an annealing temperature of 55°C and melting, annealing, and extension times of 20 s, 30 s, and 30 s for 25 cycles using Phusion polymerase. The product was purified using a Qiagen PCR purification kit, digested with NdeI and EcoRI, and ligated into the NdeI and EcoRI restriction sites of the destination vector pJK622 to yield pJK625. The plasmid encodes for H6PurS, PurQ, and PurL.

Protein expression and purification

Underlined headers indicate the final purification method. Antibiotics were used as follows: Amp (ampicillin, 100 ug/mL); Kan (kanamycin, 70 ug/mL); and Stm (streptomycin, 50 ug/mL).

a. OrfYH6

OrfYH6 (pJK530) Prep 1. A single colony of BL21(DE3) cells transformed with pJK530 was used to inoculate a 50 mL LB-Amp starter culture and grown to saturation (37°C, 200 rpm). 1 L of ZYM-5052-Amp medium [17] was inoculated 1:100 and grown overnight (37°C, 200 rpm). Cells were harvested (5,000*g*, 15 min) and stored at -80°C until use. All the following steps were performed at 4°C. The cell pellet was resuspended in 50 mM Tris-HCl pH 8.0, 300 mM KCl (5 mL/g pellet) and disrupted by sonication using a Fisher Sonic Dismembrator at 20% output (3 cycles: 1 min on, 1 min off) on ice. Cell debris was removed by centrifugation (30,000*g*, 30 min). Streptomycin sulfate (10% w/v stock) was added to a final concentration of 1% w/v, incubated on ice for 10 min, then spun (30,000*g*, 10 min). The soluble lysate was applied to a previously equilibrated and charged Ni-NTA column (1.5 cm x 2 cm, 3.5 mL) in the above buffer. The column was washed with 10 mM imidazole in the above buffer. A linear imidazole gradient (10-500 mM in 50 mM Tris-HCl pH 8.0, 300 mM KCl) was used to elute the protein (35 mL x 35 mL). Fractions containing pure protein by SDS-PAGE were pooled and the protein was concentrated using an Amicon Centricon YM-3 (7,000 rpm, 2 h). The protein was dialyzed overnight against 100 volumes (1 L) of 50 mM Tris-HCl pH 8.0, 300 mM KCl using 7,000 MWCO Pierce Snakeskin dialysis tubing (no changes). Aggregates of the dialyzed protein (flaky precipitates) were removed by centrifugation in an Eppendorf 5415D microfuge (max speed, 10 min, at 4°C). Protein quantitation was determined by the Bradford assay (Biorad). 33 mg of OrfYH6 was isolated with a final protein concentration of 7.5 mg/mL. 1 mg of OrfYH6 was buffer exchanged into i. 50 mM Tris pH 8.0, ii. 50 mM Tris pH 9.0, iii. 50 mM CHES pH 9.0, or iv. 50 mM ammonium formate pH 4.0 to a final concentration of 2 mg/mL using an Amicon Centricon YM-3. The protein was stored at 4°C and no precipitate was observed. This

batch of protein was used for antibody generation (Cocalico Biologicals, Inc.), circular dichroism (Elwood Mullins, Data Report 2013-01-07), and ESI-MS (described below).

OrfYH6 (pJK530) Preps 9-11. BL21(DE3) carrying pJK530 in ZYM-5052-Amp medium [17] was grown overnight (30°C, 200 rpm). Cells were harvested (5,000*g*, 15 min) and used immediately or stored at -80°. All the following steps were performed at 4°C. The cell pellet was resuspended in 50 mM Tris pH 8.0, 300 mM KCl (5 mL/g pellet) and disrupted by sonication (3 cycles, 1 min on, 1 min off) at 20-25% output using a Fisher Sonic Dismembrator. Cell debris was removed by centrifugation (30,000*g*, 30 min). Streptomycin sulfate (10% w/v stock) was added to a final concentration of 1% and the lysate was cleared by centrifugation (30,000g for 10 min). The cleared lysate was loaded onto a previously equilibrated (in 50 mM Tris pH 8.0, 300 mM KCl) and charged Ni-NTA column (1.5 cm x 2 cm, 3.5 mL) and the column was washed with 10 column volumes of 10 mM imidazole in 50 mM Tris pH 8.0, 300 mM KCl buffer. A linear imidazole gradient (10-500 mM in 50 mM Tris pH 8.0, 300 mM KCl) was used to elute the protein (15 mL x 15 mL). Fractions containing pure protein by SDS-PAGE were pooled and the protein was concentrated using an an Amicon ultrafiltration 8050 cell with a Millipore PL-5 membrane. For prep 9, OrfYH6 was concentrated to 14.0 mg/mL (49 mg total). For prep 10, protein was concentrated to 7.5 mg/mL (15 mg total). For prep 11, OrfYH6 was concentrated to 18.9 mg/mL and then diluted down to ~ 4.5 mg/mL to prevent precipitate formation (39 mg total). No aggregates were observed thus far at the various concentrations. These batches were used to set up crystal trays using the Emerald Wizard I and II solution kits (described below) and alkylation.

Periplasmic isolation of OrfYH6 (pJK530)

Prep 1. Protocol adapted from [18]. BL21(DE3) carrying pJK530 in ZYM-5052-Amp medium [17] was grown overnight (30°C, 200 rpm). Cells were harvested (5,000*g*, 15 min) and used immediately or stored at -80°. The cell pellet was resuspended in 400 mL of 30 mM Tris-HCl pH 8.0, 20% sucrose buffer (hypertonic solution), followed by the addition of EDTA pH 8.3 to 1 mM [final]. The solution was stirred slowly at room temperature for 10 min. The following steps were performed at 4°C. Cells were

collected by centrifugation (10,000*g*, 10 min). The supernatant was removed and the pellet was resuspended in ice cold 5 mM $MgSO_4$ (hypotonic solution, unbuffered) and stirred slowly on ice for 10 min. Cells were then spun down and the supernatant (periplasmic fraction) was transferred to a new tube. The periplasmic fraction was loaded onto a pre-equilibrated (in 50 mM Tris-HCl pH 8.0, 300 mM KCl) and charged Ni-NTA column (1.5 cm x 1.5 cm, 2.7 mL). The column was washed with 10 column volumes of 10 mM imidazole in 50 mM Tris-HCl pH 8.0, 300 mM KCl buffer. A linear imidazole gradient (10-400 mM in 50 mM Tris-HCl pH 8.0, 300 mM KCl) was used to elute the protein (15 mL x 15 mL).

Prep 2. Protocol adapted from [19]. The BL21(DE3)-pJK530 cell pellet was washed with 20 mL 30 mM Tris-HCl pH 8.0, 20% sucrose. The cells were harvested (8,000*g*, 5 min) and resuspended in 5 mL of the same buffer. 5 mL of 8 mM EDTA and 0.16 mg of lysozyme/mL were added to the cell resuspension and incubated on ice for 15 min. Cells were pelleted (8,000*g*, 10 min) and the supernatant (periplasmic fraction) was collected. The pellet was resuspended in 2 mL 50 mM Tris pH 8.0, using a 10 mL syringe and 18-gauge needle. The cells were frozen and thawed 3 times in dry ice + ethanol and a 25°C water bath, respectively, as described previously [19]. Cell debris was removed (8,000*g*, 10 min), and the supernatant (cytoplasmic fraction) was collected. Ammonium sulfate precipitation was performed on the periplasmic fraction as described previously: 0-35% (208 g/L); 35-55% (128 g/L); and 55-85% (215 g/L). The 35-55% cut was resuspended in a minimal amount of 50 mM Tris pH 8.0 buffer and loaded onto a pre-equilibrated and charged Ni-NTA column (1.5 cm x 1.5 cm, 2.7 mL). The column was washed with 10 column volumes of 10 mM imidazole buffer in 50 mM Tris pH 8.0. A linear imidazole gradient (10-400 mM) was used to elute the protein (15 mL x 15 mL). Fractions containing pure protein by SDS-PAGE were pooled and the protein was concentrated to 0.04 mg/mL using an an Amicon ultrafiltration 8050 cell with a Millipore PL-5 membrane.

Prep 3. Same protocol as prep 2 except the periplasmic fraction was directly loaded onto the Ni-NTA column (1.5 cm x 1.5 cm, 2.7 mL). To remove the EDTA, dialysis was

performed for 1 hr against 100 volumes (300 mL) of 50 mM Tris-HCl pH 8.0, 300 mM KCl using 7,000 MWCO Pierce Snakeskin dialysis tubing (no changes). Post-dialysis precipitate was removed by centrifugation and the supernatant (unknown protein concentration) was loaded onto the recharged and pre-equilibrated Ni-NTA column. The protein was eluted using 50 mM EDTA, 50 mM Tris pH 8.0, 100 mM KCl. Fractions containing protein by A280 were pooled. Ammonium sulfate fractionation was performed as a concentration step: 0-40% (242 g/L); 40-55% (63 g/L). The 40-55% pellet was resuspended in a minimal amount of 50 mM Tris pH 8.0, 100 mM KCl buffer.

Prep 6. Protocol adapted from [20]. All the following steps were performed at 4°C. In short, the BL21(DE3)-pJK530 cell pellet was resuspended in 100 mM Tris-acetate, 0.5 M sucrose, 5 mM EDTA (5 mL/g cell pellet). 40 uL of 2 mg/mL lysozyme (80 ug) and 500 uL of ice cold water were added to the cell resuspension and incubated on ice for 5 min. $MgSO_4$ was added to 5 mM final (7.5 uL of 1 M stock). Spheroplasts were pelleted in a microcentrifuge (max speed, 2 min) and the supernatant (periplasmic fraction) was collected. The pellet was washed with 1 mL 50 mM Tris-acetate pH 8.0, 0.25 M sucrose, 10 mM $MgSO_4$. Spheroplasts were pelleted (max speed, 2 min) and the supernatant was discarded. The pellet was resuspended in 1 mL 50 mM Tris-acetate pH 8.0, 300 mM KCl and lysed by sonication using a Misonix sonicator with microtip at 15W (Briggs lab, Program 3, 3 cycles of 5 s on, 5 s off, 5 s on). The periplasmic fraction was dialyzed against 100 volumes of 50 mM Tris-HCl pH 8.0, 300 mM KCl (4 buffer exchanges, 30 min, 100 mL) to remove the EDTA prior to loading 1.2 mL onto a Ni-NTA spin column. Spheroplast cellular debris was removed by centrifugation (max speed, 15 min). Qiagen Ni-NTA spin columns were equilibrated with 400 mL of 50 mM Tris-HCl pH 8.0, 300 mM KCl (800*g*, 2 min). 1.2 mL of dialyzed periplasmic solution and 700 uL of soluble spheroplast lysate were loaded onto the Ni-NTA spin columns (200*g*, 5 min). The spin columns were washed with 700 uL 50 mM Tris-HCl pH 8.0, 300 mM KCl, 10 mM imidazole (800*g*, 2 min). Bound protein was eluted using 200 uL 50 mM Tris-HCl pH 8.0, 300 mM KCl, 500 mM imidazole. Fractions were analyzed by SDS-PAGE and Western blot.

b. PurS

H6PurS and H6OrfY (pJK522). T. J. Kappock expressed pJK522 in BL21(DE3) cells in ZYM-5052-Amp medium [17]. All the following steps were performed at 4°C. The cell pellet was resuspended in 50 mM Tris-HCl pH 8.0, 100 mM KCl (5 ml/g pellet) and disrupted by sonication using a Fisher Sonic Dismembrator at 12% output with a microtip. Cell debris was removed by centrifugation (30,000*g*, 30 min). Streptomycin sulfate (10% w/v stock) was added to a final concentration of 1% w/v, incubated on ice for 10 min, then spun (30,000*g*, 10 min). Ammonium sulfate fractionation was performed as described above: 0-25% cut (144 g/L); 25-65% cut (265 g/L). The last cut was equilibrated overnight. The 25-65% pellet was resuspended in about twice the volume of the pellet and applied to a previously equilibrated and charged Ni-NTA column (1.5 cm x 4 cm, 7 mL) in the above buffer. The column was washed with 10 column volumes of 10 mM imidazole in the above buffer. A linear imidazole gradient (10-500 mM) was used to elute the protein (70 mL x 70 mL). Fractions containing pure protein by SDS-PAGE were pooled and the protein was stored as an 85% saturated ammonium sulfate pellet (608 g/L). 214 mg of H6PurS was isolated. The sample was to the Purdue University Campus-Wide Mass Spectrometry Center for MALDI-TOF.

Ammonium sulfate fractionation of PurS (pJK533). A single colony of BL21(DE3) transformed with pJK533 was used to inoculate a 50-mL LB-Amp starter culture and grown to saturation (37°C, 200 rpm). 1 L of ZYM-5052-Amp medium [17] was inoculated 1:100 and grown overnight (37°C, 200 rpm). Cells were harvested (5,000*g*, 15 min) and stored at -80°C until use. All the following steps were performed at 4°C. The cell pellet was resuspended in 50 mM Tris-HCl pH 8.0, 100 mM KCl (5 mL/g pellet) and disrupted by sonication using a Fisher Sonic Dismembrator at 20% output (3 cycles: 1 min on, 1 min off) on ice. Cell debris was removed by centrifugation (30,000*g*, 30 min). Streptomycin sulfate (10% w/v stock) was added to a final concentration of 1% w/v, incubated on ice for 10 min, then spun (30,000*g*, 10 min). Ammonium sulfate fractionation was performed as described above: 0-25% (144 g/L); 25-35% (60 g/L); 35-45% (62 g/L); 45-55% (64 g/L); 55-65% (66 g/L); 65-85% (143 g/L).

H6PurS (pJK620). A single colony of BL21(DE3) transformed with pJK620 was used to inoculate a 50-mL LB-Kan starter culture and grown to saturation (37°C, 200 rpm). 1 L of ZYM-5052-Kan medium [17] was inoculated 1:100 and grown overnight (37°C, 200 rpm). Cells were harvested (5,000*g*, 15 min) and stored at -80°C until use. All the following steps were performed at 4°C. The cell pellet was resuspended in 50 mM Tris-HCl pH 8.0, 100 mM KCl (5 mL/g pellet) and disrupted by sonication using a Fisher Sonic Dismembrator at 20% output (3 cycles: 1 min on, 1 min off) on ice. Cell debris was removed by centrifugation (30,000*g*, 30 min). Streptomycin sulfate (10% w/v stock) was added to a final concentration of 1% w/v, incubated on ice for 10 min, then spun (30,000*g*, 10 min). The cleared lysate was loaded onto a previously equilibrated and charged Ni-NTA column (1.5 cm x 1.5 cm, 2.7 mL) and the column was washed with 10 column volumes of 10 mM imidazole in 50 mM Tris-HCl pH 8.0, 100 mM KCl buffer. A linear imidazole gradient (10-500 mM) was used to elute the protein (5 mL x 5 mL). Fractions containing pure protein by SDS-PAGE were pooled and the protein was concentrated to 5.7 mg/mL using an an Amicon ultrafiltration 8050 cell with a Millipore PL-5 membrane.

c. PurQ

Co-expression of PurQ with pREP4-GroESL. C41(DE3)-pREP4-GroESL were transformed with pJK555 and used to inoculate a 50-ml LB-Amp culture which was grown to saturation. Two 250 mL cultures of ZYM-5052-Amp medium[17] were inoculated 1:50 and grown overnight at 30°C or 37°C (200 rpm). For the 15°C auto-induction, the culture was first grown at 37°C for two hours and then moved to 15°C for overnight growth and induction. Cells were harvested (5,000*g*, 15 min) and stored at -80°C until use. All the following steps were performed at 4°C. The cell pellet was resuspended in 50 mM Tris-HCl pH 8.0, 100 mM KCl (5 mL/g pellet) and disrupted by sonication using a Fisher Sonic Dismembrator at 20% output (3 cycles: 1 min on, 1 min off) on ice. Cell debris was removed by centrifugation (max speed, 15 min) in an Eppendorf microfuge 5415D at 4°C.

Inclusion body prep of PurQ (pJK555). Protocol adapted from [21]. BL21(DE3) cells

transformed with pJK555 were used to inoculate 1 L of ZYM-5052-Amp medium [17] at 1:200 and grown for 24 hours (37°C, 200 rpm). All the following steps were performed at 4°C. Cells were harvested (5,000*g*, 15 min) and used immediately or stored at -80°C until use. The cell pellet was resuspended in 50 mM Tris pH 8.0, 100 mM KCl (5 mL/g pellet) and disrupted by sonication using a Fisher Sonic Dismembrator at 20% output (3 cycles: 1 min on, 1 min off) on ice. Cell debris was removed by centrifugation (30,000*g*, 30 min). The pellet was resuspended in 100 mM Tris HCl pH 7.0, 5 mM EDTA, 15 mM βME, 2 M urea, 2% Triton X-100 (5 mL/g) using transfer pipets (Wash 1). The solution was centrifuged (30,000*g*, 30 min). 2 additional washes were performed as described previously. The final washing step was the same as above except for the buffer did not contain any urea or Triton X-100. 4 different extracting conditions were used at room temperature: 6 M guanidine-HCl, 8 M guanidine-HCl, 6 M urea, and 8 M urea. The pellet from the final wash was split up into 4 aliquots and resuspended in 50 mM Tris HCl, 5 mM EDTA, 15 mM βME, and urea or guanidine-HCl (4 mL/g). The solutions were centrifuged (30,000*g*, 30 min). The guanidine-HCl samples were subjected to ethanol precipitation to avoid precipitation when running SDS-PAGE: 225 uL of cold 100% ethanol was added to 25 uL of sample and vortexed. After 10 min at -20°C, the solution was pelleted at max speed in a microcentrifuge for 10 min. The ethanol was removed and 25 uL of H2O and 225 uL of cold 100% ethanol was added to the pellet. After resuspension, the solution was pelleted at max speed for 5 min. The pellet was resuspended in 25 uL 2X SDS buffer prior to loading.

d. PurL

H6PurL (pJK622). A single colony of BL21(DE3) transformed with pJK622 was used to inoculate a 50-mL LB-Amp starter culture and grown to saturation (37°C, 200 rpm). 1 L of ZYM-5052-Amp medium [17] was inoculated 1:100 and grown overnight (37°C, 200 rpm). Cells were harvested (5,000*g*, 15 min) and stored at -80°C until use. All the following steps were performed at 4°C. The cell pellet was resuspended in 50 mM Tris-HCl pH 8.0, 100 mM KCl (5 mL/g pellet) and disrupted by sonication using a Fisher Sonic Dismembrator at 20% output (3 cycles: 1 min on, 1 min off) on ice. Cell debris was removed by centrifugation (30,000*g*, 30 min). Streptomycin sulfate (10% w/v stock)

was added to a final concentration of 1% w/v, incubated on ice for 10 min, then spun (30,000*g*, 10 min). The cleared lysate was loaded onto a previously equilibrated and charged Ni-NTA column (1.5 cm x 1.5 cm, 2.7 mL) and the column was washed with 10 column volumes of 10 mM imidazole in 50 mM Tris-HCl pH 8.0, 100 mM KCl buffer. A linear imidazole gradient (10-500 mMin 50 mM Tris-HCl pH 8.0, 100 mM KCl) was used to elute the protein (5 mL x 5 mL). Fractions containing pure protein by SDS-PAGE were pooled and the protein was concentrated to 3.4 mg/mL using an an Amicon ultrafiltration 8050 cell with a Millipore PL-30 membrane.

PurLH6 (pJK575). A single colony of BL21(DE3) transformed with pJK575 was used to inoculate a 50-mL LB-Amp starter culture and grown to saturation (37°C, 200 rpm). 1 L of ZYM-5052-Amp medium [17] was inoculated 1:100 and grown overnight (37°C, 200 rpm). Cells were harvested (5,000*g*, 15 min) and stored at -80°C until use. All the following steps were performed at 4°C. The cell pellet was resuspended in 50 mM Tris-HCl pH 8.0, 100 mM KCl (5 mL/g pellet) and disrupted by sonication using a Fisher Sonic Dismembrator at 20% output (3 cycles: 1 min on, 1 min off) on ice. Cell debris was removed by centrifugation (30,000*g*, 30 min). Streptomycin sulfate (10% w/v stock) was added to a final concentration of 1% w/v, incubated on ice for 10 min, then spun (30,000*g*, 10 min). The cleared lysate was loaded onto a previously equilibrated and charged Ni-NTA column (1.5 cm x 1.8 cm, 3.2 mL) and the column was washed with 10 column volumes of 10 mM imidazole in 50 mM Tris-HCl pH 8.0, 100 mM KCl buffer. A linear imidazole gradient (10-500 mM in 50 mM Tris-HCl pH 8.0, 100 mM KCl) was used to elute the protein (15 mL x 15 mL). Fractions containing pure protein by SDS-PAGE were pooled and the protein was concentrated to 3.2 mg/mL using an an Amicon ultrafiltration 8050 cell with a Millipore PL-30 membrane.

PurSQLH6 (pJK606). A single colony of BL21(DE3), C41(DE3)-pREP4-GroESL, C41(DE3) transformed with pJK606 was used to inoculate a 50-mL LB starter culture and grown to saturation (37°C, 200 rpm). BL21(DE3) cells were propagated in1 L of ZYM-5052 medium [17] was inoculated 1:100 and grown overnight (37°C, 200 rpm). C41(DE3) cells with or without pREP4-GroESL were grown in LB-IPTG. In short, 1 L

LB was inoculated 1:100 and grown to OD_{600} ~0.4 at 37°C. The temperature was decreased to 15°C and when the OD_{600} reached 0.6, isopropyl-β-thiogalactopyranoside (IPTG) was added to a final concentration of 0.4 mM from a 1M stock and the cells were induced for 20 hrs. Cells were harvested (5,000*g*, 15 min) and stored at -80°C until use. All the following steps were performed at 4°C. The cell pellet was resuspended in 50 mM Tris-HCl pH 8.0, 100 mM KCl (3 mL/g pellet) and disrupted by sonication using a Fisher Sonic Dismembrator at 20% output (3 cycles: 1 min on, 1 min off) on ice. If metabolites were included, ATP, ADP, and/or glutamine would be spiked into cell lysates in this step to a final concentration of 1 mM. Cell debris was removed by centrifugation (30,000*g*, 30 min). Streptomycin sulfate (10% w/v stock) was added to a final concentration of 1% w/v, incubated on ice for 10 min, then spun (30,000*g*, 10 min). The cleared lysate was loaded onto a previously equilibrated and charged Ni-NTA column (1.5 cm x 1.5 cm, 2.7 mL) and the column was washed with 1-5 column volumes of 10 mM imidazole in 50 mM Tris-HCl pH 8.0, 100 mM KCl buffer. A linear imidazole gradient (10-500 mM in 50 mM Tris-HCl pH 8.0, 100 mM KCl) was used to elute the protein (5 mL x 5 mL) or an EDTA elution using 50 mM EDTA in 50 mM Tris-HCl pH 8.0, 100 mM KCl buffer. Batch purification using Qiagen Ni-NTA columns were used according to the manufacturer's instruction instead of using the 1.5 cm x 1.5 cm column.

Co-expression studies of AaFS proteins. LB starter cultures containing appropriate antibiotic (Tables 3-6) were inoculated with single colonies and grown to saturation (37°C, 200 rpm). Empty vector controls included pET23a, pET-Duet, or pCDF-Duet vectors. 250 mL of ZYM-5052 medium [17] supplemented with appropriate antibiotics was inoculated 1:100 and grown overnight (30°C, 200 rpm). Cells were harvested (5,000*g*, 15 min) and stored at -80°C until use. All the following steps were performed at 4°C. Cell pellets were resuspended in 50 mM Tris-HCl pH 8.0, 100 mM KCl (5 mL/g pellet) and disrupted by sonication using a Fisher Sonic Dismembrator at 20% output (3 cycles: 1 min on, 1 min off) on ice. Cell debris was removed by centrifugation (30,000*g*, 30 min or max speed, 15 min in a microfuge).

Table 1.3 Single-gene constructs (Figure 1.8, top)

pJK	Vector	Antibiotic	Gene(s)	S	Y	Q	L
531	pET23d	Amp	*orfY*		x		
555	pET23a	Amp	*purQ*			x	
533	pET23d	Amp	*purS*	x			
539	pET23a	Amp	*purL*				x

Table 1.4 Duet vector constructs (Figure 1.8, bottom)

pJK	Vector	Antibiotic	Gene(s)	SL	YQ	SYQL	SQL	QL
532	pET28a	Kan	*H6purQ*				x	
543	pCDF-Duet	Stm	*purS* and *purL*	x		x	x	x
548	pET-Duet	Amp	*orfY* and *purQ*		x	x		
549	pCDF-Duet	Stm	*purL*					x

Table 1.5 Overlapping gene constructs as in the native *A. aceti* sequence (Figure 1.9)

pJK	Vector	Antibiotic	Gene(s)	SYQL	SYQLH6	SQL	SQLH6	SYQL*	SYQLH6*
567	pCDF-Duet	Stm	*orfY*					x	x
598	pET23a	Amp	*purS-orfY-purQ-purL*	x					
599	pET23a	Amp	*purS-orfY-purQ-purLH6*		x				
605	pET23a	Amp	*purS-purQ-purL*			x		x	
606	pET23a	Amp	*purS-purQ-purLH6*				x		x

*OrfY was expressed *in trans*

Table 1.6 *purQ-purL* constructs (Figure 1.10)

pJK	Vector	Antibiotic	Gene(s)	QL	QLH6
610	pET23a	Amp	*purQ-purL*	x	
611	pET23a	Amp	*purQ-purLH6*		x

Mass Spectrometry

ESI-MS. OrfYH6 from prep 1 was analyzed on a Thermo Scientific LTQ (Parker laboratory) with Steven Ouellette's assistance. Samples were diluted by volume in 50:50:01 water: acetonitrile: formic acid. MS peaks were analyzed using ESIprot1.0 [22].

Tandem-MS. OrfYH6 was analyzed by Anton Iliuk (Tao laboratory) as follows. The protein sample was denatured and reduced in 50 mM trimethyl ammonium bicarbonate. Three sample preparations were performed: (i) 0.1% RapiGest (Waters) and 5 mM dithiothreitol (DTT); (ii) 0.1% RapiGest; or (iii) untreated (where both RapiGest and

DTT were omitted). Rapigest and/or DTT treatments were incubated for 30 minutes at 50°C. Only the sample in condition (i) was further alkylated in 15 mM iodoacetamide for 1 hour in the dark at room temperature. All samples were digested with proteomics grade trypsin at 1:100 ratio overnight at 37 °C. The pH was adjusted below 3 and the sample was incubated for 40 minutes at 37 °C. The sample was centrifuged down at 16,100*g* to remove RapiGest and to collect supernatant. The sample was desalted with a 100-mg Sep-Pak C18 column (Waters) and dried completely. Peptide samples were re-dissolved in 8 uL of 0.1% formic acid and injected into an Agilent nanoflow 1100 HPLC system. The reverse phase C18 was performed using an in-house C18 capillary column packed with 5 μm C18 Magic beads resin (Michrom; 75 μm i.d. and 12 cm of bed length) on an 1100 Agilent HPLC. The mobile phase buffer consisted of 0.1% HCOOH in ultra-pure water with the eluting buffer of 100% CH_3CN run over a shallow linear gradient over 60 min with a flow rate of 0.3 μl/min. The electrospray ionization emitter tip was generated on the prepacked column with a laser puller (Model P-2000, Sutter Instrument Co.). The Agilent 1100 HPLC system was coupled online with a high resolution hybrid linear ion trap orbitrap mass spectrometer (LTQ-Orbitrap XL; Thermo Fisher). The mass spectrometer was operated in the data-dependent mode in which a full-scan MS (from m/z 300-1700 with the resolution of 30,000 at m/z 400) was followed by 4 MS/MS scans of the most abundant ions. Ions with charge state of +1 were excluded. The mass exclusion time was 180 s. The LTQ-Orbitrap raw files were searched directly against *Saccharomyces cerevisiae* database with no redundant entries using SEQUEST algorithm on Proteome Discoverer (Version 1.0; Thermo Fisher). Proteome Discoverer created DTA files from the raw data with minimum ion threshold of 15 and absolute intensity threshold of 50. Peptide precursor mass tolerance was set at 5 ppm, and MS/MS tolerance was set at 0.8 Da. Search criteria included a static modification of cysteine residues of +57.0214 Da and a variable modifications of +15.9949 Da to include potential oxidation of methionines and carbamidomethylation of cysteines. Searches were performed with full tryptic digestion and allowed a maximum of two missed cleavages on the peptides analyzed from the sequence database. False discovery rates (FDR) were set for 1% for each analysis.

Western blotting

Antibody generation. Purified antigens OrfYH6 (pJK530) and PurLH6 (pJK575) were sent to Cocalico Biologicals Inc. for custom polyclonal antibody generation (Cocalico ELISA titers for anti-OrfYH6 and anti-PurLH6 showed signal at 1:10000 dilution). Antisera were tested by dot blot [23] or Western blotting as described below.

Western blot protocol. Protocol adapted from [24]. In short, soluble proteins from soluble lysates (*A. aceti* or *E. coli*) were separated by SDS-PAGE and transferred to a nitrocellulose membrane (0.2 or 0.45 μm, Osmonics, Inc.). Transfer was performed at 100V for 1 h in Towbin Buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol, 0.1 % (w/v) SDS). The membrane was blocked in TBS (100 mM Tris-HCl pH 7.5, 150 mM NaCl) with 5% (w/v) nonfat milk for 1 h, shaking at room temperature, followed by three 10 min washes in TBST (TBS with 0.1% (v/v) Tween-20). The membrane was probed for 1 h with custom polyclonal rabbit primary antibody (1:10,000 for anti-OrfYH6 antibody; 1:20,000 dilution for anti-PurLH6 antibody in TBS with 2% (w/v) nonfat milk) or anti-His H15 probe (1:50,000, Santa Cruz Biotechnology). Excess antibody was removed by three TBST washes. The membrane was then probed for 1 h with goat anti-rabbit IgG (H+L) HRP conjugate secondary antibody (1:10,000 dilution in TBS with 2% (w/v) nonfat milk, Jackson ImmunoResearch). Excess antibody was removed by three TBST washes. The membrane was soaked for 5 min in a 1:1 mixture of stable peroxide and luminol/enhancer solutions (ThermoScientific). Typically, signal was recorded by film or using a ChemiImager 5500 imaging system (Alpha Innotech) where membranes were exposed 30 s - 5 min.

The LI-COR Odyssey was also used with Brett Bishop's help (Ogas laboratory). For the LI-COR, membranes were blocked using the LI-COR Blocking buffer, incubated with custom antiserum for the primary and goat anti-rabbit IRDye 800 CW secondary antibody, and immediately visualized on the instrument after excess antibody was removed.

Dot blot. Protocol adapted from [23]. In short, typically 1-5 μg protein or lysate were spotted onto nitrocellulose membrane and let to dry. Non-specific sites were blocked by soaking the membrane in 5% (w/v) BSA or nonfat milk in TBST for 1 hr at room temperature in a Petri dish, followed by three 5 min washes in TBST. The membrane was incubated with primary antibody (diluted 1:25,000; 1:10,000; or 1: 5,000 in 0.1% BSA or 0.2% nonfat milk in TBS) for 30 min - 1 h. Excess antibody was removed by three TBST washes. Secondary antibody (anti-rabbit) was diluted 1:10,000 and incubated for 30 min at room temperature. Excess antibody was removed by three TBST washes and the signal was recorded as previously described.

Anti-OrfYH6 and anti-PurLH6 antibody purification. Protocol adapted from [25]. In short, Ni-IDA resin (~1.5 mL) was stripped using 100 mM EDTA. The resin was washed twice with water and recharged with 50 mM $CoCl_2$. Excess solution was removed and washed with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2PO_4, 1.8 mM KH_2PO_4, pH 7.2 [26]. 14 mg of OrfYH6 (prep 1) was used to bind the resin in PBS (30 min on ice with constant mixing, 10 mL total volume). No wash step was performed to remove unbound protein. 200 uL of 30% H_2O_2 was added to the resin and the latter was incubated at room temperature for 1 hr (Co^{2+} oxidation to Co^{3+} leads to a color change from pink to purple). The resin was then washed with PBS+100 mM EDTA and stored at 4°C in PBS until use. 2 mL of antiserum was incubated for 25 min with the resin on ice while mixing. The suspension was poured into a 0.75 cm diameter column (final dimensions 0.75 cm x 3.3 cm). Flowthrough was collected and the resin was washed with 10 column volumes of 20 mM Tris-HCl, pH 7.5, 150 mM NaCl (binding buffer) and 10 column volumes of 20 mM Tris-HCl, pH 7.5, 1 M NaCl (washing buffer). The antibody was eluted from the column with 5 column volumes of 100 mM Tris-acetate, pH 4, 150 mM NaCl; 5 column volumes of 100 mM acetate, pH 3, 150 mM NaCl; and 5 column volumes of 100 mM glycine-HCl, pH 2.5, 150 mM NaCl. Drops were collected in ~1 mL aliquots in tubes containing 100 uL 1M Tris-HCl pH 8.3. Fractions containing protein by A280nm (> 0.2 were collected), concentrated, and buffer exchanged into PBS using an Amicon UltraCentrifugal filter (2 mL; 30,000 MWCO). Purified antibody was stored at -20°C in 25% glycerol.

OrfYH6 Crystallization. Previously isolated OrfYH6 (prep 9 and 10) was used to set up crystallization trays by the hanging drop diffusion method using the Emerald Biosystems Wizard Kits I and II. In short, 500 uL of crystallization solution was aliquoted 24-well crystallization plates (Hampton Research). 4 uL drops were set up by mixing 2 uL of well solution and 2 uL of protein solution at various concentrations. Protein concentrations were determined by the Bradford assay after buffer exchanging into 10 mM Tris-HCl, pH 8.0 using Millipore Microcon YM-10 centrifugal concentrators. Trays were stored at the Hockmeyer building at Purdue University in the 21.5°C temperature-controlled crystallization room. Cryo solutions were made up by adding 15% w/v ethylene glycol to the mother liquor (Wizard Kit 1 Solution 13 (1.26 M ammonium sulfate (dibasic), 0.1 M sodium cacodylate/HCl pH 6.5) aka 1-13 or Wizard Kit 2 Solution 17 (2.5 M sodium chloride, 0.1 M Tris/HCl pH 7.0, 0.2 M magnesium chloride) aka 2-17. K. Sullivan dipped the crystals in cryo solution prior to freezing them in liquid nitrogen. Crystals were shot via remote data collect LS-CAT beam line 21-ID-F.

OrfYH6 alkylation with N-ethylmaleimide (NEM) and methoxypolyethylene glycol maleimide MAL-PEG. Samples that were used were unpurified periplasmic fraction containing OrfYH6, purified OrfYH6 (from total lysate, prep 11), and *A. aceti* thioredoxin (Jesse Murphy). The periplasmic fraction from cells overexpressing OrfYH6 were freeze-thawed once as described above, and the supernatant was kept for further processing. NEM was added to samples to a final concentration of 10 mM in 50 mM TrisHCl, pH 6.5. As for MAL-PEG, 5 molar equivalents over the total number of cysteines was added in 50 mM TrisHCl, pH 6.5. Samples that were first treated with DTT had 5 molar equivalents over the total number of cysteines found in OrfYH6. Excess MAL-PEG was neutralized with 5 molar equivalents of β-mercaptoethanol (BME) prior to analysis by SDS-PAGE.

1.3 Results and Discussion

ExPASy [27] predicted the sizes of PurS, OrfY, PurQ, and PurL to be 8.7 kDa, 12.9 kDa, 25.1 kDa, and 77.7 kDa, respectively. The sizes of *A. aceti* PurS, PurQ, and PurL are similar to previously characterized Gram-positive FS [5-7].

Co-expression studies of AaFS proteins

After single gene constructs were obtained, double gene constructs were made in Duet vectors where each gene was under its own T7 promoter. A preliminary overexpression of the AaFS genes is shown in Figure 1.8.

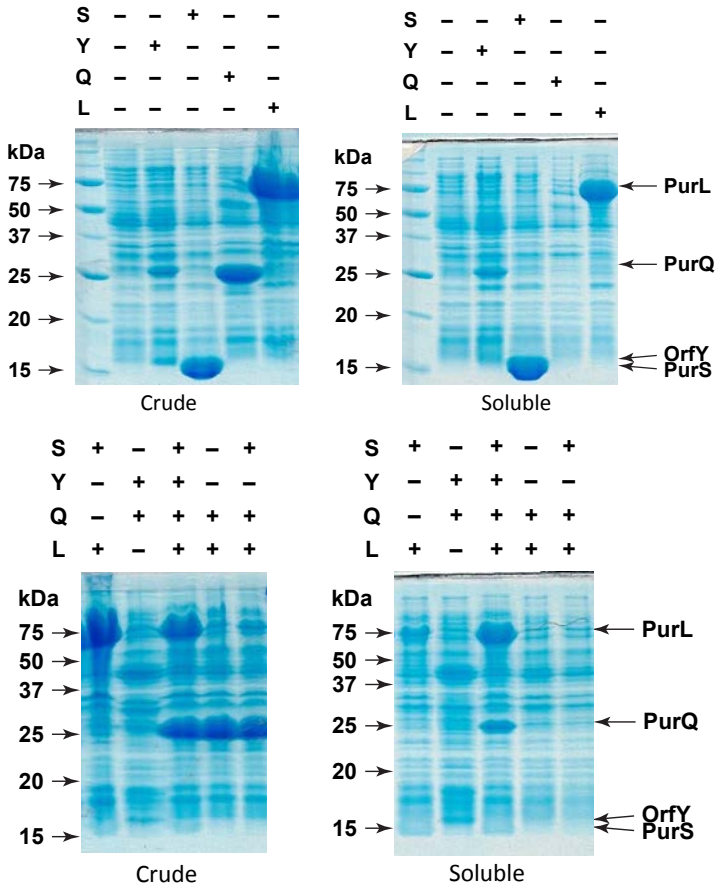

Figure 1.8 Expression of AaFS proteins.

Top, individual construct expression. Bottom, Duet vector constructs (NB - PurQ is actually H6PurQ (pJK532) in the last 2 lanes of the gels). Tables 3 and 4 in Materials and Methods lists the plasmids used for expression. SDS-PAGE (12% acrylamide), 10 uL of 1:5 dilution/lane (protein concentration unknown).

OrfY, PurS, and PurL were soluble. PurQ, like its tagged version (data not shown), was insoluble. When PurQ is co-expressed with the other three *A. aceti* genes, a portion becomes soluble, thus PurQ might rely on the other subunits of the AaFS complex or even GroESL for proper folding and therefore solubility. An OrfY dimer is visible and looks to co-migrate with PurQ when expressed alone (Western blotting in Figure 24, right, on purified OrfYH6 with purified anti-OrfYH6 antibody probing supports the dimer hypothesis). Induction failed with pJK548, which carries OrfY and PurQ on the same Duet vector.

Constructs where the overlapping nucleotides were conserved between the AaFS genes were obtained and expressed as shown in Figure 1.9. Additionally, OrfY on a separate plasmid (pJK567) was re-added to the 3-gene constructs (pJK605/606) to test if OrfY has a *cis* or *trans* effect.

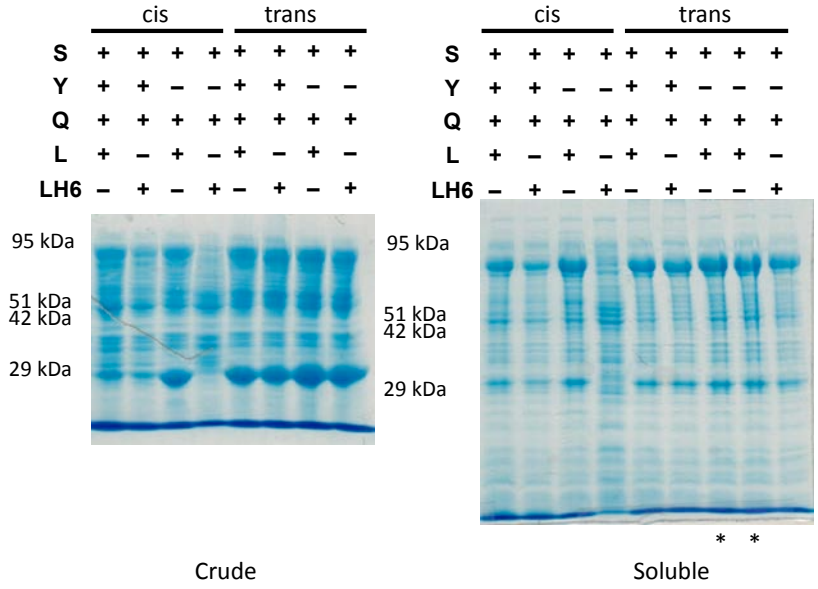

Figure 1.9 Expression of FS proteins (overlapping genes).

Table 1.5 lists the plasmids used for expression. For *trans,* pCDF-Duet was used in the -OrfY lanes (right-most lanes). * denotes duplicate sample. SDS-PAGE (12% acrylamide), 10 uL/lane after 1:10 dilution (protein concentration unknown).

pJK606 induction failed in the *cis* experiment, but OrfY was observed to have a repressive effect in *cis* on PurQ and potentially PurL. Re-addition of OrfY (in *trans*) has no effect on the over-expression of PurQ or PurL. More definitive conclusions could be drawn if the same amount of protein was loaded. A small amount of soluble PurQ was observed in the presence of PurS and PurL. QL(H6) constructs were obtained and expressed (Figure 1.10).

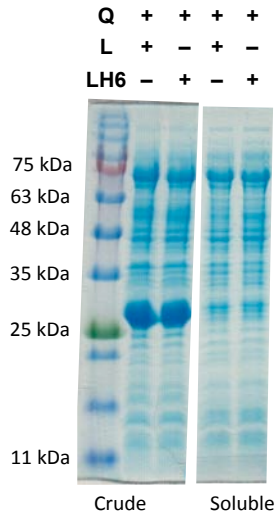

Figure 1.10 Coexpression of PurQL.

Expressed constructs were as follows: PurQL (pJK610) and PurQLH6 (pJK611). SDS-PAGE (12% acrylamide), 10 uL/lane after 1:10 dilution (protein concentration unknown).

The solubility of PurQ seems to require the presence of both PurS and PurL, as not much soluble PurQ was observed when only PurL is present.

H6PurS/H6OrfY (pJK522)

pJK522 encodes for H6PurS and H6OrfY, where the 4-nucleotide overlap was preserved as in the native sequence, despite the presence of the His-tag at the N-term of OrfY. The idea behind using pJK522 for purification was that both H6PurS and H6OrfY would be able to be purified in the same prep. 214 mg of protein was isolated. Since one band was observed by SDS-PAGE, the sample was analyzed by MALDI-TOF where only H6PurS was detected (10703.33 Da). The expected sizes for H6PurS-Met1 and H6OrfY-Met1 were 10.7 kDa and 13.8 kDa, respectively. Analysis of isolated OrfYH6

by ESI-MS below revealed that OrfY is N-terminally processed, thus explaining why only H6PurS was isolated here.

OrfYH6 (pJK530)

Prep 1. Expression of OrfYH6 showed that the protein was soluble (data not shown). The elution profile is shown in Figure 1.11.

Figure 1.11 OrfYH6 prep 1 elution profile (linear imidazole gradient).

Ladder used was bovine serum albumin (BSA, 66 kDa) and hemoglobin, Hb (Chain A, 16 kDa; Chain B, 17 kDa). SDS-PAGE (12% acrylamide), 10 uL/lane (protein concentration unknown).

Flaky precipitate was observed after overnight dialysis and was spun down. The final purification gel is shown in Figure 1.12.

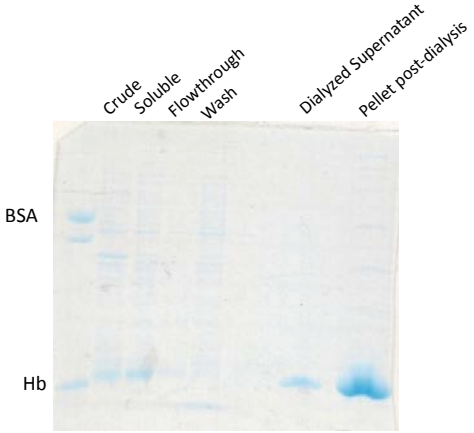

Figure 1.12 OrfYH6 final purification gel (prep 1).

SDS-PAGE (12% acrylamide); 5 ug/lane except for the pellet post-dialysis (protein concentration unknown).

The observed flaky precipitate after dialysis is likely OrfYH6 by SDS-PAGE (Figure 1.14). 33 mg of soluble protein was isolated and appeared to remain in solution when stored at 4°C in 50 mM Tris pH 8.0, 300 mM KCl at a protein concentration of 7.5 mg/mL,; 50 mM Tris pH 8.0; 50 mM Tris pH 9.0; 50 mM CHES pH 9.0; or 50 mM ammonium formate pH 4.0 at 2 mg/mL for at least one year.

Steven Ouellette (Parker laboratory) and Anton Iliuk (Tao laboratory) helped with ESI-MS analysis of OrfYH6 and after deconvolution using ESIprot 1.0, the molecular weight was determined to be 11927.45 Da (expected 14094.76 Da). SignalP 3.0 algorithm analysis [28] of the OrfYH6 sequence predicts a cleavage site between A22 and Q23 (Figure 1.13 shows those residues to be A21 and Q22 as Met1 was omitted when the protein sequence was entered into the algorithm).

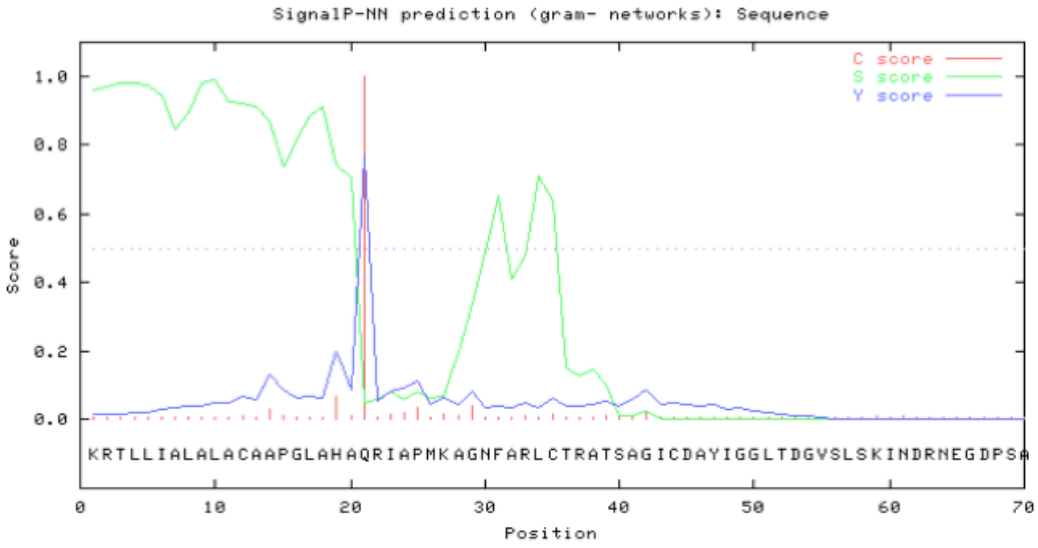

Figure 1.13 SignalP 3.0 output for the analysis of OrfYH6.
A cleavage site is predicted between A22 and Q23 (A21 and Q22 in graph as Met1 was omitted).

The SignalP 3.0 output for OrfYH6 resembles that of a secretory or periplasmic protein thus suggesting OrfYH6 secretion or export. The predicted molecular weight of processed OrfYH6 is 11935.6 Da, a few Da off from the ESI-MS experimental value of 11927.45 Da.

Elwood Mullins used circular dichroism (CD) to further characterize OrfYH6. The CD spectrum is shown in Figure 1.14 and the data summary is in Table 1.7.

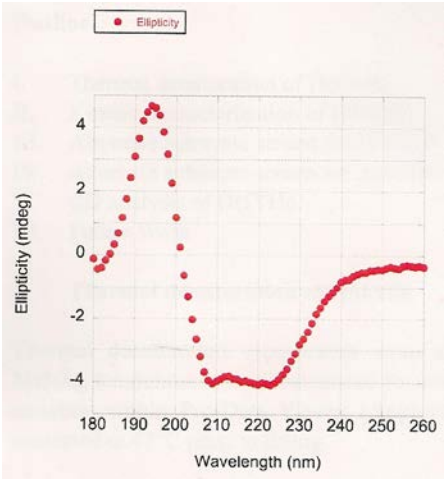

Figure 1.14 CD spectrum of OrfYH6.
Refer to EAM Data Report 2013-01-07 for experimental details.

Table 1.7 CD structural element summary for OrfYH6.

	180-260 nm	185-260 nm	190-260 nm	195-260 nm	200-260 nm	205-260 nm	210-260 nm
Helix	39.40%	40.10%	40.80%	43.60%	47.40%	50.10%	49.10%
Antiparallel	5.50%	5.10%	4.80%	5.30%	5.30%	5.00%	5.00%
Parallel	7.10%	7.00%	7.00%	6.40%	5.80%	5.40%	5.80%
Beta-turn	16.10%	15.90%	15.70%	15.10%	14.50%	14.30%	14.30%
Random coil	26.70%	26.60%	26.60%	25.40%	24.10%	23.30%	24.10%
Total sum	**94.90%**	**94.60%**	**94.90%**	**95.70%**	**97.20%**	**98.10%**	**98.40%**

According to CD, OrfYH6 is 40% α-helical while β-sheets and random coils make up the remaining 60%.

Anton Iliuk (Tao laboratory) helped with tandem MS analysis of OrfYH6. Multiple cysteine residues can be found within the OrfY protein sequence (C13, C36, C45, C76, and C119). The last four residues were found to be conserved in at least 14 other OrfY sequences as summarized in Figure 1.15.

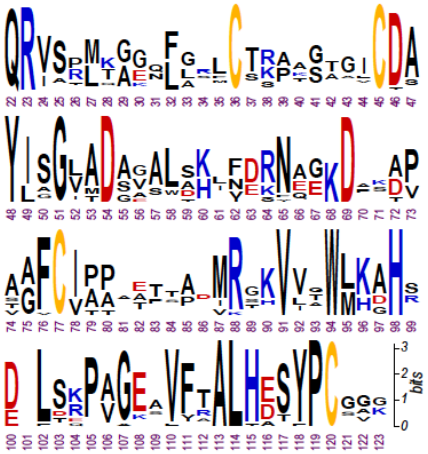

Figure 1.15 Sequence logo for the conserved residues of 14 OrfY sequences. The more highly conserved residues are larger.

OrfYH6 was analyzed by tandem-MS analysis in reducing and non-reducing conditions in attempt to identify any existing disulfide bridges (Figure 1.16).

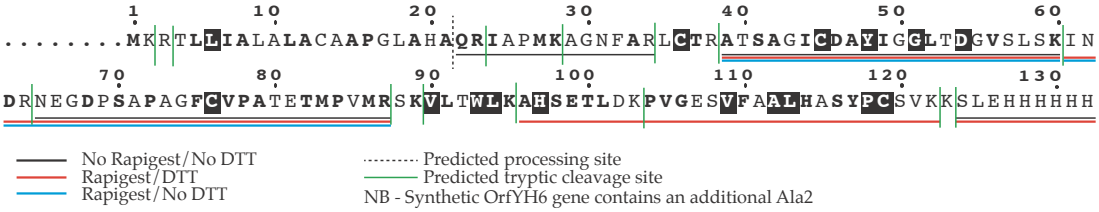

Figure 1.16 Tandem-MS peptide coverage of OrfYH6.

Detected peptides were underlined in different sample prep conditions (black - no RapiGest/no DTT; red - no RapiGest/ + DTT; blue - + RapiGest/no DTT). The vertical dashed line denotes the predicted N-term processing site and the vertical green lines, tryptic digest site.

Out of the 111 amino acids in processed OrfYH6, only 12 amino acids were not covered when the three experiments were combined (89% coverage). As expected, the first 22 amino acids (note that the accommodation of the NcoI site at the N-term leads to an additional alanine residue at position 2, which was not included in the Figure 1.18 sequence) were not detected. The first cysteine residue, C13, is part of the processed N-term. In untreated cell lysate (black underline in Figure 1.16), C45 and C72 were

detected (their reduced state may have been due to reductants found in cell lysate). In reducing conditions (red underline in Figure 1.16), C45, C72, and C119 were detected. In non-reducing conditions (blue underline in Figure 1.16), C45 and C72 were detected. For all experiments, C36 was likely on a peptide that was too small for detection. Since only the +DTT sample was alkylated with iodoacetamide, it is still unclear if all the cysteine residues in OrfYH6 are reduced.

Isolation of OrfYH6 in 100 mM KCl (vs. 300 mM KCl in prep 1) showed a poor yield of OrfYH6 as only 2 mg of protein was isolated in prep 2 (data not shown). A decrease in the auto-induction temperature from 37°C to 30° seemed to help expression in prep 3 (data not shown). A spreading out effect was observed in both the imidazole and EDTA elutions (data not shown).

Prep 9-11. The buffer conditions from prep 1 were used and a linear imidazole gradient (10-500 mM) was used to elute the protein. The final purification gel and accompanying Western blot using custom antiserum (not antigen-affinity purified yet) for prep 9 and the final purification gel for prep 10 is shown in Figure 1.17.

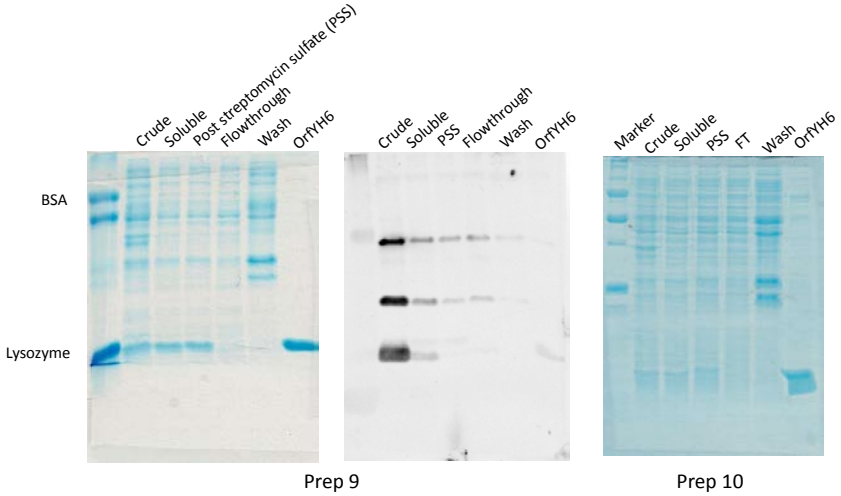

Figure 1.17 OrfYH6 preps 9 and 10 final purification gels.

Left, final gel for prep 9. Center, Western blot using custom antiserum (LI-COR). Right, final gel for prep 10. SDS-PAGE (15%), 5 ug protein/lane.

49 mg of OrfYH6 was isolated from 4L of cells for prep 9, and 15 mg from 1L for prep 10. The Western blot results were not as expected. Although OrfYH6 was detected in

the crude, soluble, and OrfYH6 lanes, two strong signals were observed at higher molecular weights. At first glance, the bands could be OrfYH6 oligomers. However due to the lack of marker signals and a very faint signal for the purified protein, possible explanations include non-specific reactivity of the antiserum to *E. coli* contaminating proteins or the latter's emission at 800 nm. Other Western blots where signal was recorded on film show non-specific reactivity of antiserum to *E. coli* empty vector lysate (Figure 1.18).

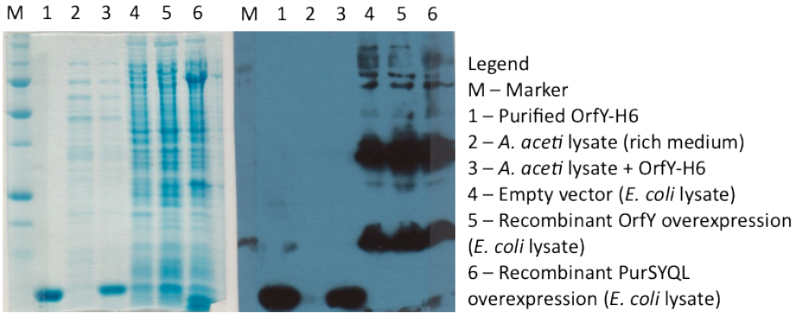

Figure 1.18 PU81 Antiserum has non-specific reactivity to *E. coli* lysate. Left, Coomassie-stained SDS-PAGE (12%), 5 ug protein/lane. Right, Western (15 s exposure).

M. Hall and N. Dudareva suggested antigen affinity purification of the antiserum to enrich for the anti-OrfYH6 antibody. Dot blot results showed interaction with OrfYH6, as expected, but also, *E. coli* and *A. aceti* lysates, likely due to an error in the blocking step where 5% BSA (w/v) was used instead of 5% nonfat milk (w/v) in TBST (data not shown). Instead of repeating the dot blot, a Western blot was performed using the purified OrfYH6 antibody and is shown in Figure 1.19. 5% nonfat milk (w/v) was used for blocking.

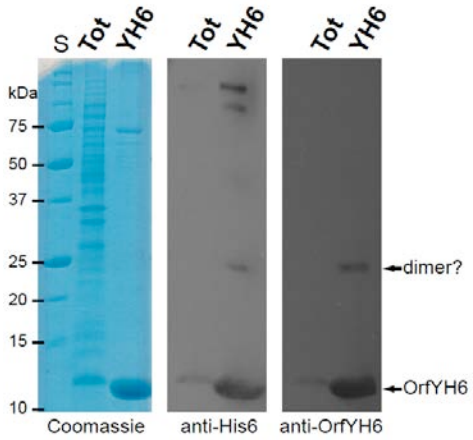

Figure 1.19 Western blot of OrfYH6 total lysate and purified OrfYH6.

Santa Cruz H-15 His probe or antigen affinity purified anti-OrfYH6 antibody were used. Left, SDS-PAGE (12% acrylamide), 5 ug protein/lane. Center, blot using anti-His antibody. Right, blot using purified anti-OrfYH6 antibody. 2 min exposure. Legend: molecular weight standard, S; total lysate, Tot; purified OrfYH6 (prep 9), YH6.

Both the anti-His and the purified OrfYH6 antibodies were able to detect monomeric OrfYH6 in total lysates, although the signal was very faint. In the purified OrfYH6 lanes, both antibodies were observed to probe for the monomeric and potentially dimeric OrfYH6. The putative OrfYH6 dimer was not observed in the total lysate lane. The antigen purified antibody seemed to work well as the anti-His antibody was observed to from non-specific interactions at very high molecular weights in the purified OrfYH6 lane.

Purified OrfYH6 from preps 9 and 10 were used to set up crystal trays. After 4 months, some crystals were observed in two buffers: (1-13) 1.26 M ammonium sulfate, 0.1 M sodium cacodylate/HCl, pH 6.5 and (2-17) 2.5 M sodium chloride, 0.1 M Tris/HCl pH 7.0, 0.2 M magnesium chloride.

Tray 1 Well C1 (NB3-137, 2012-06-15) with crystallization buffer 1-13 contained a single crystal with 2 faces and was also not birefringent and Izit negative. This crystal was dubbed “OrfY1” and had the following unit cell parameters, and OrfYH6 being a potential hexamer:

Unit cell dimensions in A, deg(o) and cubic A :						
a	b	c	alpha	beta	gamma	volume
219.7000	219.7000	219.7000	90.000	90.000	90.000	10604499.0

Space group F 2 3 , space group number 196, Laue class m-3 , z= 48

System as decoded from unit cell dimensions : cubic
is consistent with space group F 2 3 : cubic

The F 2 3 space group calculated for OrfY2 is not common, therefore crystallization conditions should be optimized in 1.26M ammonium sulfate, 0.1M sodium cacodylate/HCl, pH 6.5 for OrfYH6 for further crystallographic studies.

OrfYH6 prep 11, which yielded 39 mg of protein from 3 L of cells (Figure 1.20), was used for NEM and MAL-PEG alkylation of cysteine residues (Figure 1.21).

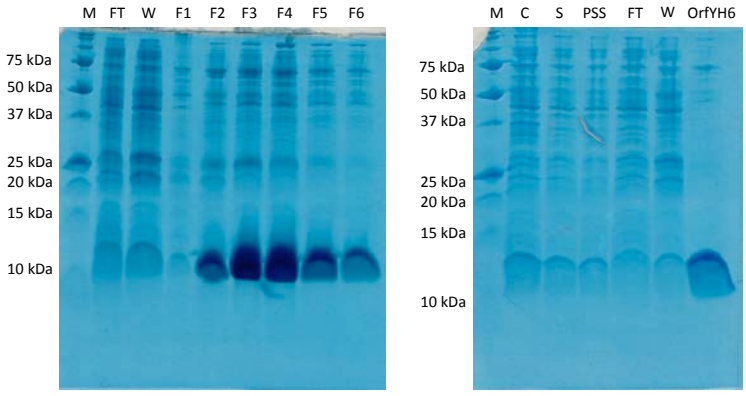

Figure 1.20 OrfYH6 prep 11.

Left, elution gel (10 uL/lane, protein concentration unknown). Right, final purification gel (5 ug protein/lane). SDS-PAGE (15% acrylamide).

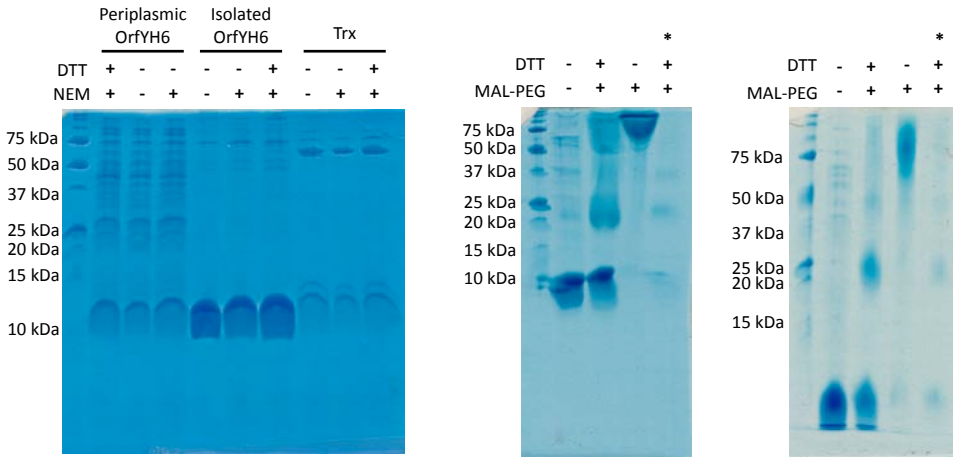

Figure 1.21 Alkylation of OrfYH6 with NEM and MAL-PEG.

Left, NEM alkylation (SDS-PAGE, 15%; 10 uL/lane). Center and right, alkylation of isolated OrfYH6 with MAL-PEG (SDS-PAGE, 15% urea and 9% urea, respectively; 7.5 ug protein/lane). * indicates sample was purified with a Ni-NTA spin column.

Successful NEM and MAL-PEG modification of cysteine residues in the absence of DTT would indicate reduced cysteines. On the other hand, if no shift is observed in the absence of DTT, the cysteine residues are likely to be oxidized. Regardless of the state of the cysteines (oxidized or reduced), a shift should be observed in the presence of both DTT and alkylator. The NEM treatment did not yield a large enough shift to be convincing by SDS-PAGE (Figure 1.21, left) so MAL-PEG was used on purified OrfYH6 (Figure 1.21, center and right) since it would add ~ 5 kDa per modified cysteine. SDS-PAGE urea gels were used to hopefully get better band resolution; however urea does not seem to help as smears and fuzzy bands were observed. Since 12% gels led to OrfYH6 migrating to the very bottom of the gel (Figure 1.18) and 15% caused smearing (Figure 1.20), 13.5% gels should be used. Previous studies have reported that MAL-PEG alkylation caused a greater mass shift of 10-15 kDa than the expected 5 kDa by SDS-PAGE [29]. OrfYH6 treated with MAL-PEG in the absence of DTT showed a smear at ~ 25 kDa. In reducing conditions, a smear was observed at higher molecular weights, however it is unclear how many MAL-PEG residues are attached to OrfYH6 (Figure 1.21, right). Together with the tandem MS data obtained thus far (Figure 1.16) and the unresolved SDS-PAGE alkylated bands, it is still unclear if the OrfYH6 residues are

reduced or not. More samples and new gels (*i.e.* 13.5% acrylamide) will have to be used to resolve the different molecular weight species due to alkylation.

An attempt at isolating MAL-PEG-modified OrfYH6 (no DTT) using Ni-NTA spin columns failed (Figure 1.21, right, last lane) in order to eventually use that method to concentrate OrfYH6 isolated from the periplasm. One reason is that a small amount of protein was input (45 ug protein) onto the spin-column for recommended elution volume (>200 uL).

Periplasmic Isolation of OrfYH6

OrfY was predicted to be an exported protein by SignalP 3.0 (Figure 1.13) and ESI-MS data supports N-term processing of OrfYH6. Many, but not all, secreted and/or periplasmic proteins undergo N-term processing. To test this hypothesis, OrfYH6 periplasmic isolations were attempted.

In prep 1, cells were lysed by osmotic shock [18]; however, the OrfYH6 in the periplasmic fraction was extremely dilute, well below the level of detection by SDS-PAGE and Coomassie staining (data not shown). Lysozyme was used in another periplasmic isolation and was utilized in prep 2, followed by a freeze-thaw protocol [19]. The final purification gel is shown in Figure 1.22.

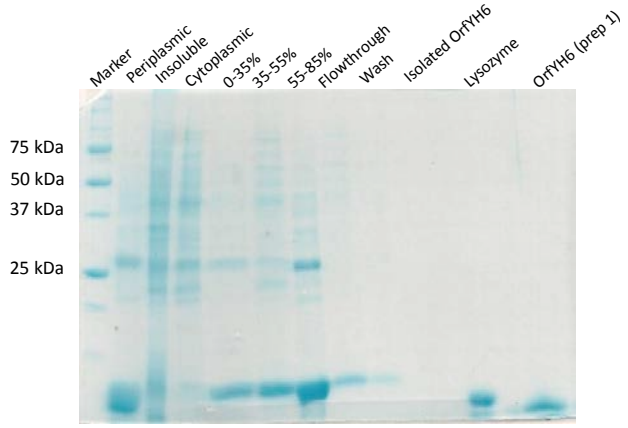

Figure 1.22 Periplasmic isolation of OrfYH6 using lysozyme (prep 2). SDS-PAGE (12%); 10 ug/lane except for "Wash" (3 ug) and "Isolated OrfYH6" (1 ug).

Once again, the isolated OrfYH6 was very dilute and below the detection level of Coomassie staining. Lysozyme and OrfYH6 are very close in size and were observed to migrate closely on SDS-PAGE (Figure 1.22). A higher percentage gel may be useful for better resolution of both proteins as it is unclear if OrfYH6 is present at all; a Western blot was performed using an anti-His antibody to determine the presence of OrfYH6 (Figure 1.23).

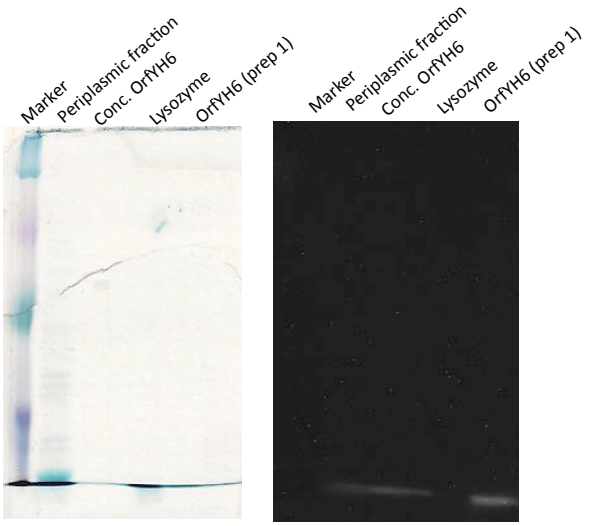

Figure 1.23 Western blot visualization of periplasmic OrfYH6.
Santa Cruz H-15 anti-His probe was used. Left, SDS-PAGE (9% acrylamide), 10 ug/lane. Left, chemiluminescence signal after 1 min exposure.

OrfYH6 migrated with the dye front; however, successful probing of periplasmic OrfYH6 and previously purified protein was observed. As expected, lysozyme was not detected by Western blot. The Western protocol seemed to work well and periplasmic OrfYH6 was isolated although very dilute. Switching some of the steps around may increase yield. In prep 3, ammonium sulfate was performed after running the affinity column; however, there was still 4 mM EDTA, which was enough to strip the Ni-NTA column. The flowthrough was dialyzed and the affinity purification was repeated. As in the OrfYH6 prep 1, flaky precipitate after overnight dialysis (no buffer changes) was observed so dialysis should be avoided. No signal by Western blot was observed in preps 3 and 4.

Prep 5. The protocol for prep 3 was attempted again since no OrfYH6 was detected by Western blot. OrfYH6 was once again very dilute and was not visualized on SDS-PAGE by Coomassie staining (data not shown). A Western was performed using anti-His and the custom antisera from Cocalico (Figure 1.24).

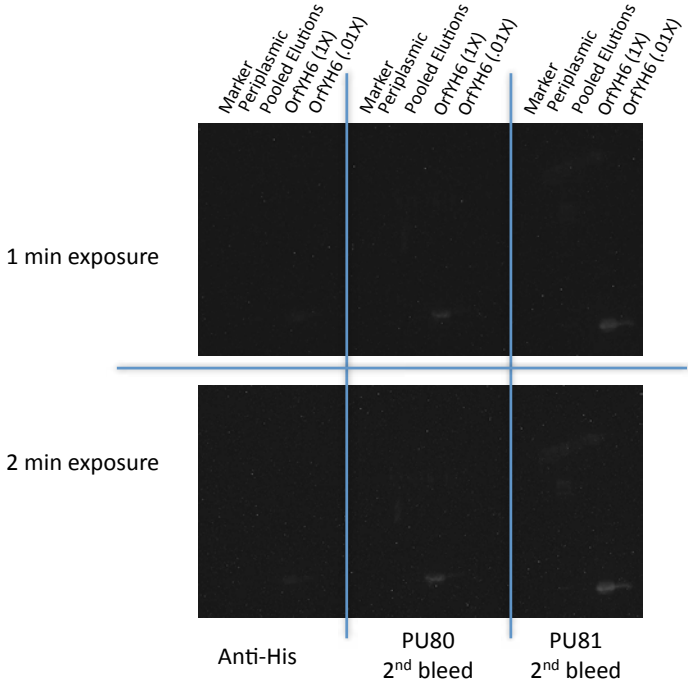

Figure 1.24 Western blot for the periplasmic isolation of OrfYH6 prep 5. Different primary antibodies were used: left, anti-His (Santa Cruz); center, custom antiserum from PU80 rabbit (Cocalico); right, custom antiserum from PU81 rabbit (Cocalico). The last 2 lanes denoted "OrfYH6" were from prep 1.

The secondary bleed antiserum from Cocalico (note that this periplasmic prep was performed before the OrfYH6 antibody was purified) were able to successfully detect purified OrfYH6 but not the periplasmic form likely due to exported OrfYH6 being too dilute.

Prep 6. Issues encountered in previous preps included using too much lysozyme and a very dilute periplasmic fraction, both of which contributed to visualizing OrfYH6 difficult by SDS-PAGE. Cells were resuspended in a smaller amount of buffer (5 mL/g cell pellet) and treated with less lysozyme (80 ug) [20].

The gels for the periplasmic purification attempt and Western are shown in Figure 1.25.

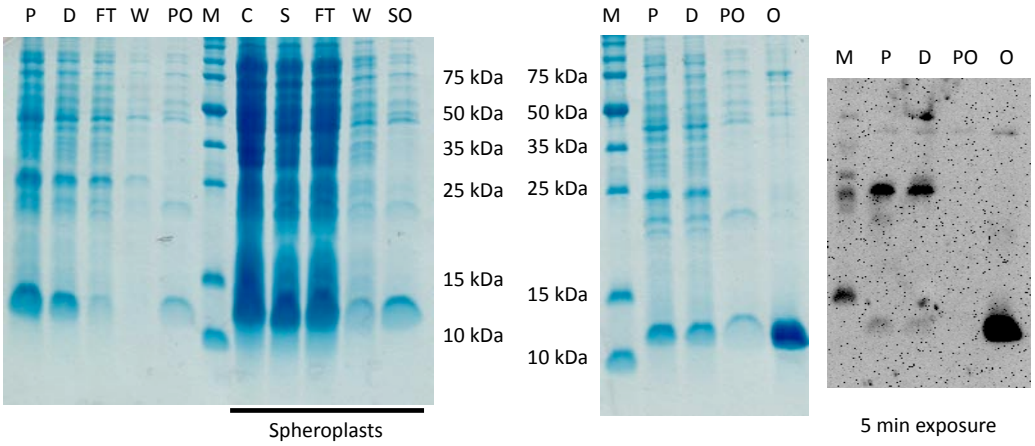

Figure 1.25 Periplasmic isolation of OrfYH6 prep 6.

Left, isolation of OrfYH6 from periplasmic and spheroplasts (periplasmic fraction: 12 ug; periplasmic OrfYH6: 6 ug; spheroplast solubly lysate: 22 ug; spheroplast OrfYH6: 6 ug; other lanes: protein concentration not determined). Center, Coomassie gel for Western blot analysis of periplasmic fractions for OrfYH6 (5 ug protein/lane). Right, Western blot detection of OrfYH6 (5 ug protein/lane) using the purified anti-OrfYH6 custom antibody (Cocalico), 5 min exposure. SDS-PAGE (13.5% acrylamide). Legend: M - marker; P - periplasmic fraction; D - dialyzed periplasmic fraction; FT - flowthrough; W - wash; C - crude; S - soluble; SO - spheroplast (cytoplasmic) OrfYH6; PO - periplasmic OrfYH6; O - purified OrfYH6 from total lysate (prep 11).

The amount of lysozyme loaded onto the SDS-PAGE gels in Figure 1.25, left, was determined to be 1.5 ug in the periplasmic fraction lane. By SDS-PAGE, it is unclear if the protein band at ~ 12 kDa is OrfYH6 or lysozyme, but it is present in all the lanes, including the spheroplasts (cytoplasmic) fractions with the exception of the periplasmic wash. Previous experience with the periplasmic fraction was the inability to detect much protein since it was so dilute. A Western blot was performed on the periplasmic, dialyzed periplasmic, purified periplasmic OrfYH6 and previously purified OrfYH6 from total cells (prep 11) as a positive control (Figure 1.25, center and right). The anti-OrfYH6 antibody was found to have some non-specific interaction with the protein marker; however, as expected, a strong signal was observed for the isolated monomeric OrfYH6 (Figure 25, right, last lane, band at ~ 12 kDa). A strong signal at ~25 kDa, which could correspond to a putative OrfYH6 dimer, was detected by the purified anti-

OrfYH6 antibody in the pre and post-dialysis periplasmic fraction (Figure 1.25, right), but the monomeric form of OrfYH6 was not detected. The SDS-PAGE loading buffer contained 300 mM BME yet the dimer was resistant to denaturing and reducing conditions. One hypothesis is that OrfYH6 exists as a dimer in the periplasm and binds a metabolite (maybe iron?). No signal for the monomeric or the dimeric forms of OrfYH6 were observed after the Ni-NTA spin column was used (Figure 1.25, right, PO lane), which suggests that the His tags of OrfYH6 in the dimer failed to interact with the resin. It is important to note that isolated OrfYH6 from total lysate has yielded the monomeric form, although a faint dimer was observed by SDS-PAGE in crude/soluble lysates (Figure 1.8). It has not yet been determined if the dimer flowed through and/or came out in the wash (flowthrough and wash have not been tested by Western blot). One possible explanation for the OrfYH6 dimer failing to interact with the Ni-NTA resin is that the His-tags become buried upon dimer formation and are no longer solvent-exposed. A larger tag (*i.e.* GST) could be used however its size may interfere with dimer formation.

PurSQLH6 (pJK606)

B. subtilis FS, composed of PurS, PurQ, and PurL, was purified and assembled *in vitro* [7] in the presence of small metabolites. If AaFS behaves like BsFS, the complex could be co-purified using the pJK606 construct since it encodes for PurS, PurQ, and PurLH6. Prep 1 buffers did not contain any metabolites. Although not much PurL or PurQ was expressed, some PurLH6 and PurQ enrichment was observed (data not shown); however, since the buffer only contained 100 mM KCl, higher salt may help with maintaining electrostatic interactions between subunits.

C41(DE3) carrying the pREP4-GroESL and pJK606 constructs successfully expressed GroEL and GroES but failed to produce PurSQLH6 (data not shown). PurS could have migrated with GroES in the dye front. A band migrating at ~ 25 kDa was observed and could correspond to PurQ (data not shown); however, there still is work to be done in optimizing expression of pJK606.

Batch purifications using the Ni-NTA spin columns proved unsuccessful as well (data not shown) mainly due to the small amount of starting recombinant protein. A quick and dirty attempt where a larger pellet was processed and a Ni-NTA column was used for co-purification in the presence of metabolites is shown in Figure 1.26.

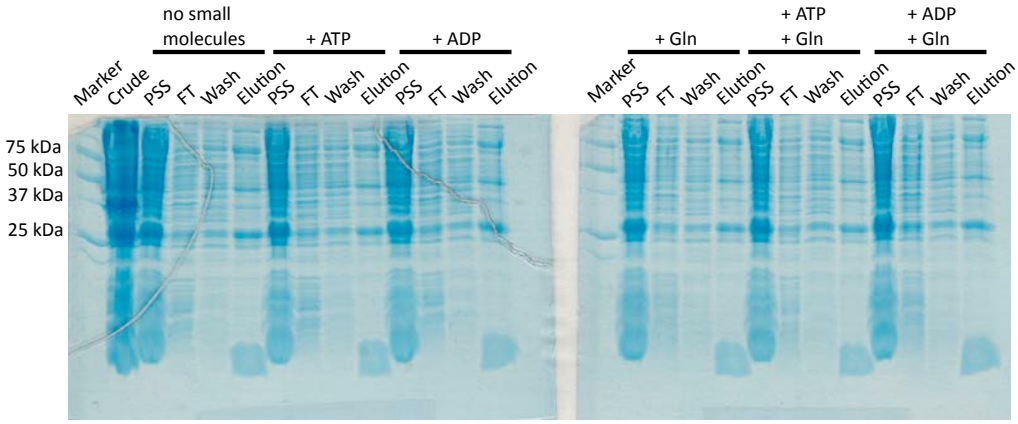

Figure 1.26 PurSQLH6 pulldown in the presence of metabolites.
SDS-PAGE (15% acrylamide), 10 uL/lane undiluted (protein concentration unknown).
Legend: PSS - post streptomycin sulfate, FT - flowthrough.

The elution fractions were dirty, due to the small volume of wash buffer applied (1 column volume), but bands that could correspond to PurS, PurQ and PurL were able to be visualized. A 15% acrylamide gel was used here for better visualization of PurS. The presence of small metabolites ATP, ADP, glutamine, or combinations of those did not seem to improve complex formation for co-isolation. PurS seemed to be enriched compared to PurQ and PurL, maybe due to the 2 PurS : 1 PurQ : 1 PurL as previously described in BsFS [3, 7] and TmFS [5]. Since PurQ was found to be insoluble even in the presence of PurL (Figure 1.9), characterization of AaFS may best be achieved by kinetic studies of a *ΔpurL* BL21(DE3) strain overexpressing pJK605 (PurSQL) or pJK606 (PurSQLH6).

Ammonium sulfate fractionation of PurS (pJK533). The salting out profile of PurS is shown in Figure 1.27.

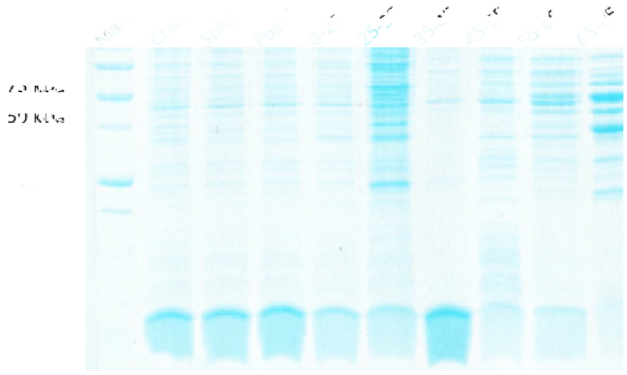

Figure 1.27 Ammonium sulfate fractionation of PurS (pJK533).
SDS-PAGE (15%), 5 ug protein/lane. The six right-most lanes are the ammonium sulfate cuts (numbers denote percentages).

PurS seems to precipitate out of solution in all cuts; however, the cleanest and most highly concentrated (at 26 mg/mL) cut was the 35-45% cut, which will be used in subsequent purifications. No further work on untagged PurS was performed.

H6PurS (pJK620). Some H6PurS was isolated from the pJK522 construct, however, pJK522 also coded for H6OrfY, which was not isolated due to an N-terminal truncation (consistent with ESI-MS data). The N-terminal His tag on PurS should not affect FS complex formation as crystal structures show it to be solvent exposed even when PurS was crystallized alone [5, 30]. The pJK620 construct only encodes for H6PurS. The elution and final purification gels are shown in Figure 1.28.

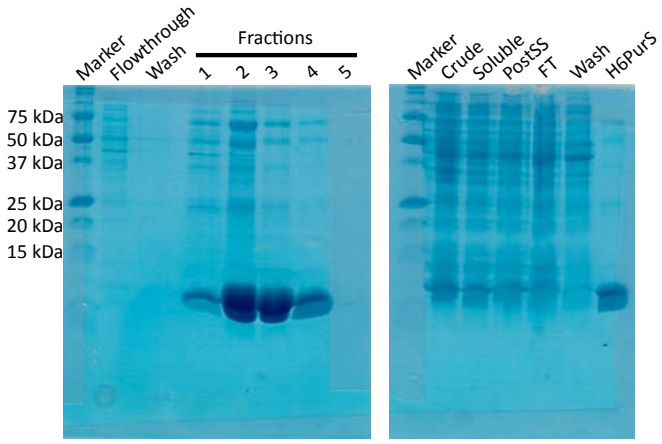

Figure 1.28 Purification of H6PurS (pJK620).

Left, elution profile, 10 uL/lane. Right, final purification gel, 5 ug/lane. SDS-PAGE (15%).

Fractions 2 and 3 were pooled and a total of 20 mg H6PurS was isolated from 1 L of cells. No further characterization has been done for H6PurS.

PurQ.

Co-expression of PurQ with pREP4-GroESL. In the preliminary co-expression studies above, PurQ was observed to be produced in large amounts but as an insoluble protein. Co-expression of PurQ with pREP4-GroESL may help solubilize PurQ. Different expression temperatures were tested and the gel is shown in Figure 1.29.

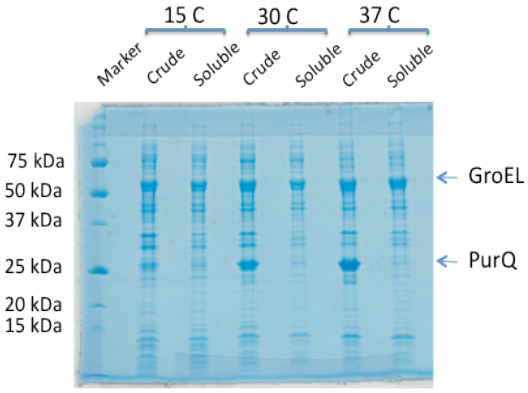

Figure 1.29 Co-expression of PurQ (pJK555) with plasmid pREP4-GroESL.

Protein expression was tested at 15°C, 30°C, and 37°C. SDS-PAGE (12%), 10 uL/lane after a 1:10 dilution.

The presence of GroESL does not seem to improve PurQ solubility regardless of the growth temperature. Other alternatives to solubilize PurQ are inclusion body preps or fusion constructs.

Inclusion body prep of PurQ (pJK555). Co-expression of PurQ with GroESL failed to improve PurQ solubility so inclusion body prep was attempted (Figure 1.30).

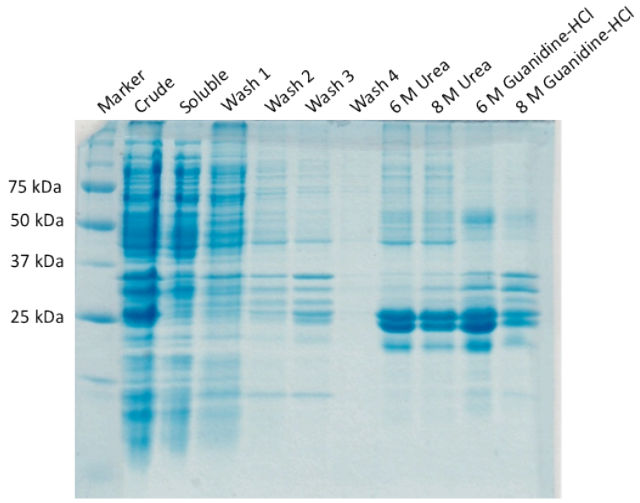

Figure 1.30 Inclusion body prep of PurQ (pJK555).
SDS-PAGE (12%), 10 uL/lane (crude and soluble samples diluted 1:5).

Extraction was successful and it looks like inclusion body prep will work for PurQ, although the presence of double bands may be due to proteolytic cleavage. Refolding of denatured PurQ was not attempted.

PurL

H6PurL (pJK622). Protein-protein interaction interfaces between PurS, PurQ, and PurL are currently unknown for the AaFS complex. The TmFS complex shows that both the C and N-termini of PurL are solvent exposed [5]. The elution and final purification gels are shown in Figure 1.31.

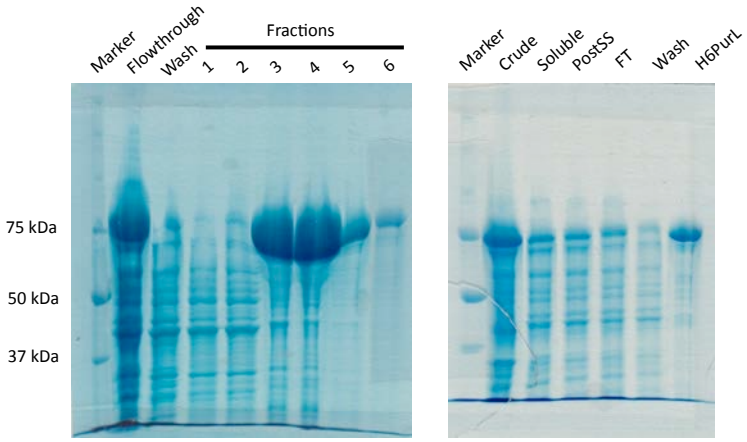

Figure 1.31 Purification of H6PurL (pJK622).

Left, elution gel, 10 uL/lane (protein concentration unknown). Right, final purification gel, 5 ug/lane. SDS-PAGE (9% acrylamide).

Fractions 3 and 4 were pooled. The column was overloaded as a lot of unbound H6PurL was observed in the flowthrough. 11 mg of protein was isolated with some lower molecular weight contaminating proteins, however a bigger column should be used for future isolations.

PurLH6 (pJK575). The C-terminally tagged PurL was also isolated and the gels are shown in Figure 1.32

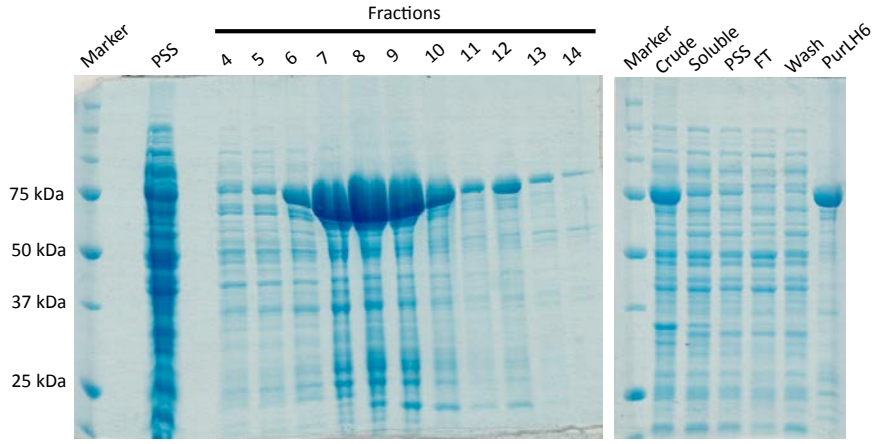

Figure 1.32 Purification of PurLH6 (pJK575).

Left, elution profile, 10 uL/lane. Right, final purification gel, 5 ug/lane. SDS-PAGE (9%). Legend: PSS - post streptomycin sulfate; FT - flowthrough.

Fractions 7-9 were pooled and 12 mg were isolated. This prep was sent to Cocalico Biologicals in order to get a custom antibody made for PurLH6. As for the OrfYH6 antibody, PurLH6 antiserum was affinity purified and tested via dot blot (Figure 1.33).

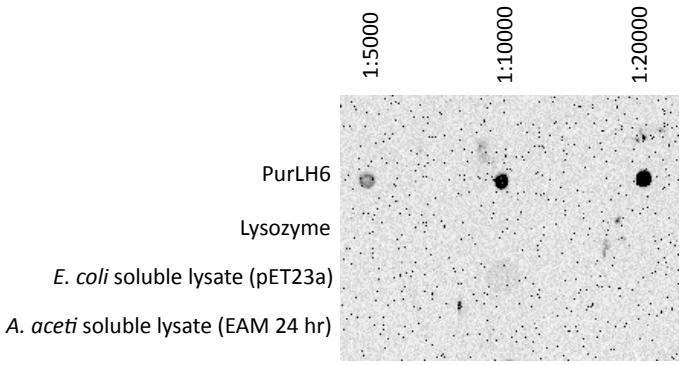

Figure 1.33 Dot blot for purified PurLH6 antibody.

4 ug each of PurLH6, lysozyme, *E. coli*, and *A. aceti* lysates were spotted on nitrocellulose membrane. 30 min exposure using the ChemImager. Image is reverse of original and auto-contrast was used.

The Prozone effect was observed, where a stronger signal was observed at the lower dilution (1:20,000) than at the higher dilution (1:5,000). According to J. Whitesell (Cocalico), the ELISA results also reflect this effect. The Prozone effect [31] occurs when there is more antibody than there is antigen, the antibody of interest cannot attach to the antigen due to the presence of other antibodies so a lower signal is observed at the lower dilution than at the higher dilution. The purified antibody will be used at a 1:20,000 dilution and it has not yet been tested for Western blots. The purified antibody will not only be able to be used for Westerns but also pull-down of the AaFS complex, although more studies on AaFS complex formation need to be performed.

1.4 Conclusion

OrfYH6 was isolated on multiple occasions from total cell lysate and was observed to be monomeric and stable at 4°C for over a year in 50 mM Tris pH 8.0, 300 mM KCl at a concentration of 4.3 mg/mL to 7.5 mg/mL. Overnight dialysis of OrfYH6 (from total cells or periplasmic) is not recommended as flaky precipitate was observed

the next morning. The higher amount of salt (300 mM KCl) in the buffer led to a greater yield of purified OrfYH6. From nickel affinity chromatography, the majority of the isolated species was observed to be monomeric by reducing and denaturing SDS-PAGE; the Western blot in Figure 1.19 suggests that some OrfYH6 dimer was detected in the purified fraction. ESI-MS data supported the prediction of an N-terminally processed protein; however further work needs to be performed in isolating OrfYH6 in the periplasm so the identity of the cysteine residues (oxidized or reduced) and why the dimer is visible by reducing SDS-PAGE can be elucidated. Current hypotheses are that OrfYH6 may contain disulfide bonds or that it could be involved in binding iron for iron-sulfur cluster biogenesis due to the proximity of *bolA* and *grx* (Chapter 4). The dimer was also detected by Western blot in the periplasmic OrfYH6 isolation prep 6 (Figure 1.25) and potentially rules out the disulfide hypothesis since the dimer subunits would become monomeric in reducing SDS-PAGE sample buffer. Conclusions cannot be drawn at this time for the oxidized or reduced states of the cysteines within OrfYH6 from tandem-MS and MAL-PEG alkylation data. Optimization for the enrichment of the periplasmic OrfYH6 dimer should consider the possibility of the C-term His-tags being buried upon dimer formation as no signal at ~ 25 kDa was detected by Western blot in the Ni-NTA resin elution fraction (Figure 1.25).

Tagged FS proteins (H6PurS, H6PurL, and PurLH6) have been isolated except for H6PurQ which was found to be insoluble like the untagged protein. None of the untagged AaFS proteins were isolated, but some preliminary work was done for the ammonium sulfate fractionation of PurS (pJK533).

Further work will have to be performed on the co-purification of the AaFS complex. Preliminary results suggest that the PurSQL complex may not require metabolites (ATP, ADP, and/or glutamine) to assemble; however due to the low amount of starting recombinant protein, protein expression for the pJK606 construct may have to be optimized. Enzymatic characterization of the active AaFS complex may be performed in a *ΔpurL* BL21(DE3) strain as an alternative to purifying the complex; however determination of the subunit stoichiometry will require purification. PurQ insolubility

will make the purification of the individual subunit difficult and unlike BsFS, the presence of PurL does not drive PurQ into the soluble fraction significantly [7]. Functional complementation studies (Chapter 2) have determined that functional AaFS is composed of PurS, PurQ, and PurL, although the stoichiometry of the subunits is still unknown. If enough AaFS can be co-purified, gel filtration or Edman degradation and crystallization could be performed to define the subunit composition stoichiometry of AaFS.

Anti-OrfYH6 and anti-PurLH6 antibodies were generated and will be useful to detect OrfY and PurL in *A. aceti* crude lysates, where their expression levels may be low, depending on the culture conditions, which have yet to be developed for *A. aceti* (preliminary work in Chapter 5). For Westerns, the final dilutions for probing were 1:10,000 for the anti-OrfYH6 and 1:20,000 for the anti-PurLH6 antibodies. The antibodies could also be used for immunoprecipitation of any interacting partners (for example, OrfYH6 as an iron-sulfur chaperone would interact with iron-sulfur cluster target proteins).

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CHAPTER 2. FUNCTIONAL COMPLEMENTATION STUDIES OF AAFS

2.1 Introduction

Previous characterizations of FS have revealed the existence of a complex of single domain proteins versus one large multi-domain protein, both of which are able to catalyze the same reaction, the conversion of FGAR, ATP, and L-Gln to FGAM, ADP, P_i, and L-Glu [1-7]. Gene cluster alignments suggest that AaFS is composed of PurS, PurQ and PurL (Chapter 1); however the presence of *orfY* is unexpected. OrfY can be found in the genomes of other α-proteobacteria but to date, there are no characterized OrfY orthologs. This chapter focuses on functional complementation studies of the AaFS genes in a *ΔpurL E. coli* strain.

2.2 Materials and Methods

Materials

All materials and chemicals were from Sigma Aldrich or Fisher Scientific and of the highest purity unless otherwise noted. *E. coli* Keio JW2541 (*ΔpurL::kan*^{R}) and parent (wild-type) BW25113 strains were obtained from the Keio Collection [8]. pET vectors were from Novagen and oligodeoxynucleotides (ODNs) were from IDT.

Table 2.1 Oligodeoxynucleotides used in this chapter.

ODN	Sequence (5'=>3')[a]
2146	GTTCGTAATCCAGGCCAAAA
2147	ATGCTAACCGAGGGGAAGTT
2154	CTGTATTTTCAGGGCGGATCtATGATGGAAATTCTGCGTG
2155	CACGCAGAATTTCCATCATaGATCCGCCCTGAAAATACAG

[a]Letters shown in lower case code for mutagenesis.

Table 2.2 Plasmids used in this chapter[a].

pJK	Vector	Description	Source
530	pET23d	*orfYH6*	Chapter 1
531	pET23d	*orfY*	Chapter 1
532	pET28a	*purQ* (silA63)	Chapter 1
533	pET23d	*purS*	Chapter 1
539	pET23a	*purL*	Chapter 1
543	pCDF-Duet	*purS* and *purL*	Chapter 1
544	pCDF-Duet	*purS*	Chapter 1
546	pET-Duet	*orfY*	Chapter 1
548	pET-Duet	*orfY* and *purQ*	Chapter 1
549	pCDF-Duet	*purL*	Chapter 1
555	pET23a	*purQ*	Chapter 1
556	pET22bHT	*EcH6purL*[b]	This study
567	pCDF-Duet	*orfY*	Chapter 1
598	pET23a	*purS-orfY-purQ-purL*[a]	Chapter 1
599	pET23a	*purS-orfY-purQ-purLH6*[a]	Chapter 1
605	pET23a	*purS-purQ-purL*[a]	Chapter 1
606	pET23a	*purS-purQ-purLH6*[a]	Chapter 1
610	pET23a	*purQ-purL*[a]	Chapter 1
623	pET23a	*orfY-purQ-purL*[a]	Chapter 1
674	pET22bHT	*EcH6purL2*	[9]
NA	pCP20	*FLP recombinase*	[8]

[a]Hyphens indicate overlapping genes (as in the native *A. aceti* sequence). [b]*Ec* indicates the *E. coli* gene. NA - not applicable

Media were supplemented with antibiotics in the final concentrations as follows: Amp (ampicillin, 100 ug/mL) for overnight cultures or Cb (carbenicillin, 100 ug/mL) for long-term cultures; Kan (kanymycin, 70 ug/mL); streptomycin (50 ug/mL).

Repaired *E. coli H6purL* construct plasmid pJK556. QuikChange mutagenesis (Qiagen) was performed on pJK674 [9] to repair a frame shift mutation using ODNs 2154 and 2155.

Preparation of competent cells. Keio JW2541 cells were made competent for transformation as previously described [10].

Keio strain JW2541 kanamycin cassette pop-out. The kanamycin cassette was removed by homologous recombination as previously described [11] by transforming pCP20 [12] into the strain of interest. In short, pCP20-transformed JW2541 cells were plated and grown for 42 hrs at room temperature on LB-Amp. The pCP20 plasmid was obtained from Charles Constantine's -20°C box and no glycerol stocks were made from the intermediate strains. A single colony was picked to inoculate a 5 mL LB culture (no antibiotics) and grown for 4 hrs at 42°C to induce expression of FLP [12]. After making a 1:1000 dilution, 50 uL of cells were plated on LB, LB-Amp, and LB-Kan, to test for loss of antibiotic resistance and of the plasmid. The plates were incubated at 42°C overnight. To further confirm loss of the kanamycin cassette to generate Keio JW2541 Kan^S, colony PCR was performed on colonies from the LB plate using ODNs 2146 and 2147.

Complementation on M9 minimal medium. Procedure adapted from [13, 14]. Keio JW2541 Kan^R and Kan^S were transformed with pET23a, AaFS plasmids, or pJK674 [9]. For growth on solid M9 medium, a single colony was used to inoculate a 5 mL LB culture and grown at 37°C to OD_{600} ~ 0.5 (~3 hr). Cultures were diluted to a final OD_{600} of 0.0005 and 50 uL was plated on M9 agar plates supplemented with antibiotic(s)/thiamin or antibiotic(s)/thiamin/hypoxanthine (10 uM thiamin and 1.5 ug/mL hypoxanthine final). Antibiotics are listed in Table 2.3. For growth in liquid M9 medium, single colony was used to inoculate a 5-mL LB culture and grown at 37°C to OD_{600} ~ 0.5 (~3 hr). Cells were harvested at 4,000 rpm, 10 min and washed with M9 medium. 250 mL Klett flasks containing 50 mL of M9 minimal medium (supplemented with thiamin or thiamin and hypoxanthine) were inoculated to a final OD_{600} of 0.005.

The cultures were grown at 37°C (200 rpm) and growth was monitored using a Klett-Summerson colorimeter (blue filter #42, spectral range 400-465 nm). No other filter was available at the time.

Table 2.3 Antibiotic list used in the M9 medium.

		Antibiotics		
Plasmid(s)	JW2541	Kan	Cb	Stm
No plasmid	Kan^R	+	-	-
pET23a (Empty Vector, EV)	Kan^R	+	+	-
544 (*purS*) + 531 (*orfY*)	Kan^S	-	+	+
544 (*purS*) + 532 (*purQ*)	Kan^S	+	-	+
543 (*purS* and *purL*)	Kan^S	-	-	+
548 (*orfY* and *purQ*)	Kan^S	-	+	-
530 (*orfY*) + 549 (*purL*)	Kan^R	+	+	+
532 (*H6purQ*) + 549 (*purL*)	Kan^R	+	-	+
544 (*purS*) + 548 (*orfY* and *purQ*)	Kan^S	-	+	+
543 (*purS* and *purL*)+ 531 (*orfY*)	Kan^S	-	+	+
548 (*orfY* and *purQ*) + 549 (*purL*)	Kan^S	-	+	+
532 (*H6purQ*) + 543 (*purS* + *purL*)	Kan^S	+	-	+
543 (*purS* and *purL*) + 548 (*orfY* and *purQ*)	Kan^R	+	+	+
539 (*purL*)	Kan^R	-	+	-
533 (*purS*)	Kan^S	-	+	-
531 (*orfY*)	Kan^S	-	+	-
532 (*H6purQ*)	Kan^S	+	-	-

+ denotes use, - omission. ^{R} resistant, ^{S} sensitive.

Complementation on MOPS minimal medium. Growth medium was prepared as previously described [15, 16]. The M9 protocol was followed as described above using JW2541 Kan^s but 1X MOPS was used to wash the cells twice. For the second liquid complementation (8 hr intervals), the green filter #54 (spectral range 500-570 nm) was used for a closer approximation to typical $O.D._{600\ nm}$ readings. All media were supplemented with Amp or Cb since only overlapping gene constructs for AaFS genes were used (Table 2.2).

96-well plate format (MOPS). Nina Serratore helped with setting up a 96-well plate growth assay on the Briggs lab Biotek Synergy 4 heated microplate reader. Each well contained 150 uL of media (n=3 for each strain ± hypoxanthine) and 2xMOPS-washed cells were used to inoculate the wells to a final $O.D._{600\ nm}$ of 0.005. The plate that was used was a sample from MidSci (Brand*plates*(R) pureGrade S, Ref. 781660, U-bottom). The instrument parameters were for a Grainer 96 round bottom plate, set point temperature of 37°C, shake: fast (continuously), (A) 600 nm, and read every 2 hrs. Blank and WT wells did not contain any carbenicillin; however all wells contained thiamin. Unused wells were filled with 150 uL sterile water and the sides of the plate were parafilmed to minimize evaporation effects.

2.3 Results and Discussion

Complementation in M9. Keio JW2541 Kan^S and Kan^R were used for the complementation on M9 medium (solid or liquid). Since 2 different strains were used those results were not comparable. Additionally the cells were not washed so some growth was observed on all plates, even on the negative control, pET23a. Despite those mistakes, the major trend was that the addition of thiamin and hypoxanthine helped growth. Studies of liquid cultures would better resolve the differences between growth phases better in the different constructs (*e.g.* lag phase). Klett flasks were used in order to minimize contamination opportunities as the flasks do not need to be open to take aliquots for turbidity readings. In the liquid cultures, pJK674 [9], which was used as a positive control, did not complement as well as expected (data not shown), and sequencing revealed a base insertion at position 51 (right after the N-term His-tag), multiple silent mutations (G729, G761, G769, L770, S803, V811, P817, A1043, V1044, and V1223), and a N869E missense mutation, the latter which seems to be located on the surface of the protein (based on the *Salmonella typhimurium* PurL structure (PDB: 1T3T [6]). The frame-shift was repaired by site-directed mutagenesis to yield pJK556. It is important to note that the wild type *E. coli* BW25113 strain was not tested on M9 medium.

Complementation in MOPS Minimal Medium. The recipe for M9 does not contain metal supplements (*i.e.* iron) whose presence may be necessary for growth so MOPS minimal medium [15] was chosen. Figure 2.1 shows the preliminary MOPS growth curves.

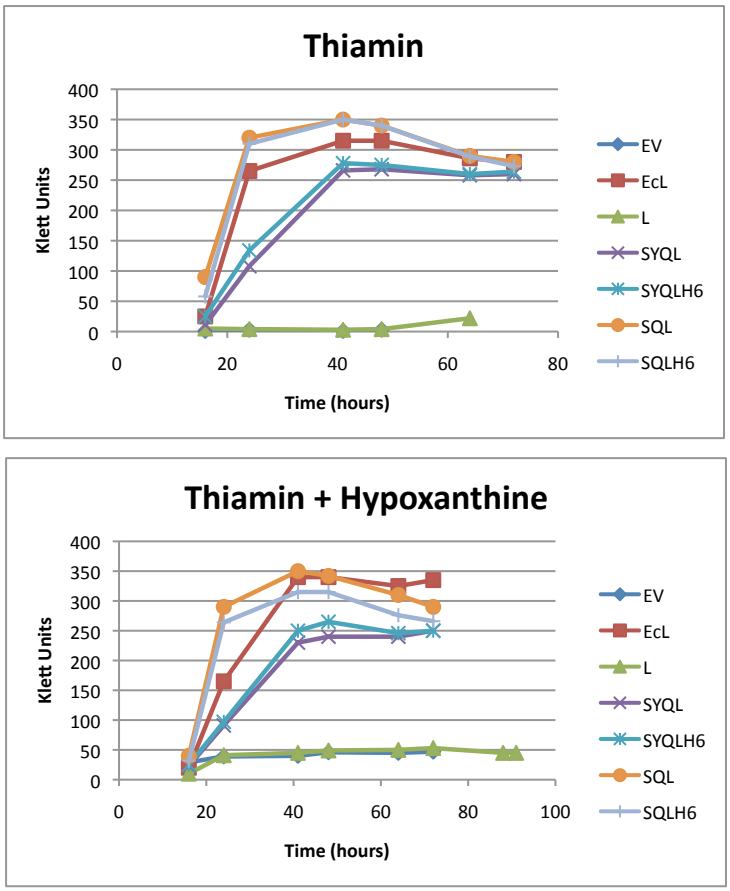

Figure 2.1 Preliminary MOPS growth curves.

Constructs used were pET23a (empty vector, EV), pJK556 (EcH6L, labeled EcL); pJK539 (AaL); pJK598 (SYQL); pJK599 (SYQLH6); pJK605 (SQL); and pJK606 (SQLH6).

The *E. coli* H6PurL (pJK556) successfully complemented the deletion strain in the thiamin only medium. Cells transformed with the empty vector pET23a showed poor growth in thiamin only medium (the curve is hidden underneath AaL, *A. aceti* PurL alone), but it should be noted that the culture touched the foil lid at 48 hours therefore time points thereafter were omitted due to possible contamination. Cells carrying the PurS-PurQ-PurL (SQL) construct grew as well if not better than the *E. coli* H6PurL. *A.*

aceti PurL showed very poor complementation. The presence of the C-term His tag on PurL did not affect its ability to complement the *purL* deletion in JW2541 for the constructs containing PurS-PurQ-PurL (SQL) and PurS-OrfY-PurQ-PurL (SYQL). It is important to note that the wild type *E. coli* BW25113 strain was not tested here.

Functional AaFS seems to be composed of PurS, PurQ, and PurL as the SQL and EcH6PurL lag phases were very similar. The stoichiometry of the subunits required for a functional AaFS complex cannot be determined from these functional complementation studies. The presence of OrfY seems to delay reaching stationary phase and is not necessary for FS complementation as observed by the growth of SQL being similar to that of *E. coli* H6PurL. The presence of a His-tag at the C-term of AaPurL does not seem to affect complex formation and complementation ability. The observed growth of AaL after 64 hrs may be due to high enough concentrations of free ammonia in the medium. Previous *B. subtilis in vitro* characterization determined that small PurL had 50-fold less activity when enough ammonia was in the assay buffer compared to PurL complexed with PurS and PurQ [2].

The growth curves were repeated once the QL and YQL constructs were obtained (pJK 610 and pJK 623, respectively), with monitoring at 8 hr intervals in MOPS minimal medium are summarized in Figure 2.2 (error bars included) and Figure 2.3 (error bars omitted). Figures 2.4 and 2.5 show the unaveraged curves for each strain. It is important to note that the wild type *E. coli* BW25113 strain did not grow when monitored for 80 hrs in thiamin or thiamin and hypoxanthine-supplemented MOPS medium (one biological replicate, data not shown).

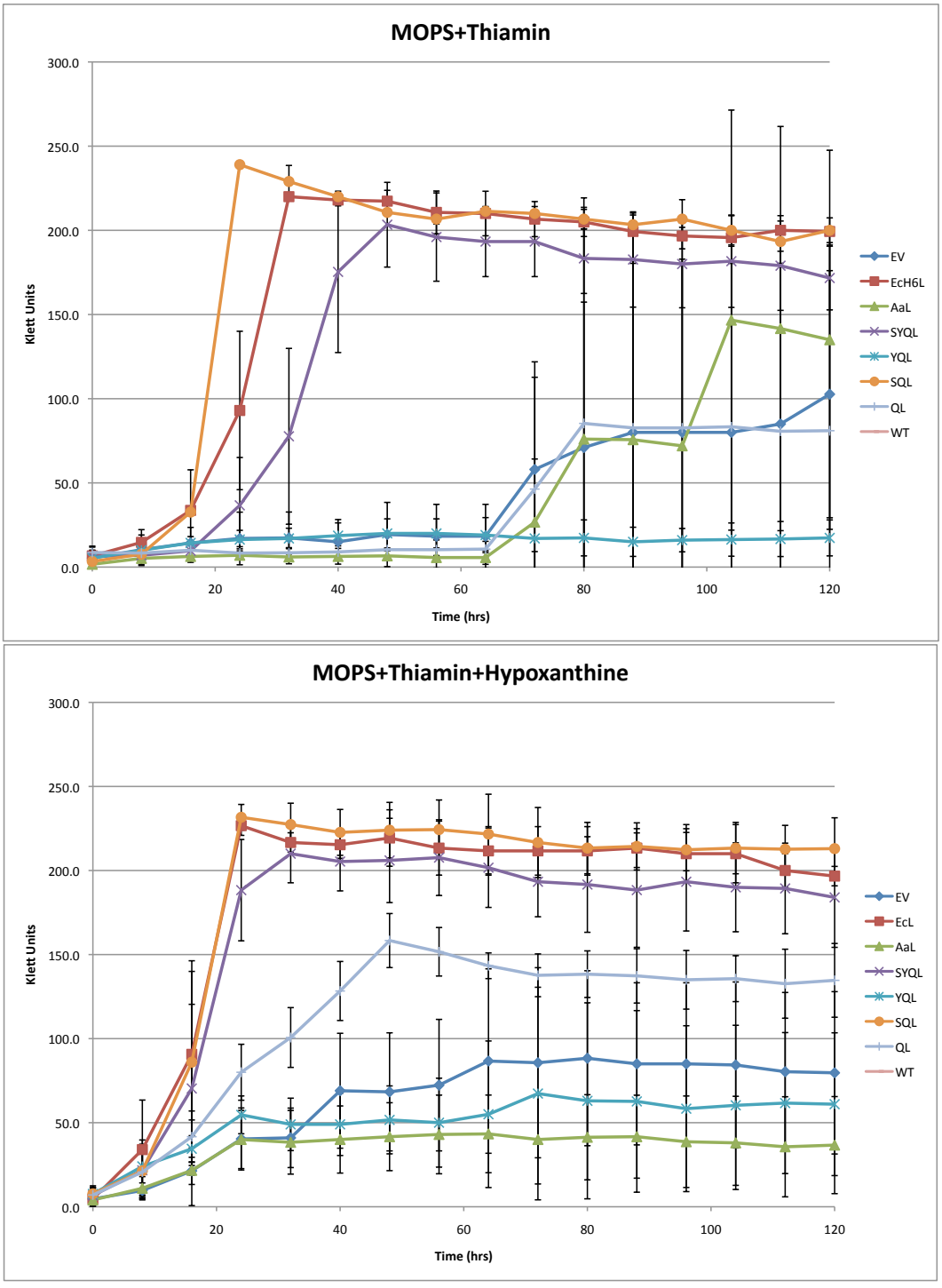

Figure 2.2 Functional complementation of AaFS proteins in JW2541 Kan^{s} in MOPS minimal medium including error bars.

Biological replicates were n = 3. Constructs used were pET23a (EV), pJK556 (EcH6L); pJK539 (AaL); pJK598 (SYQL); pJK605 (SQL); pJK610 (QL); and pJK623 (YQL).

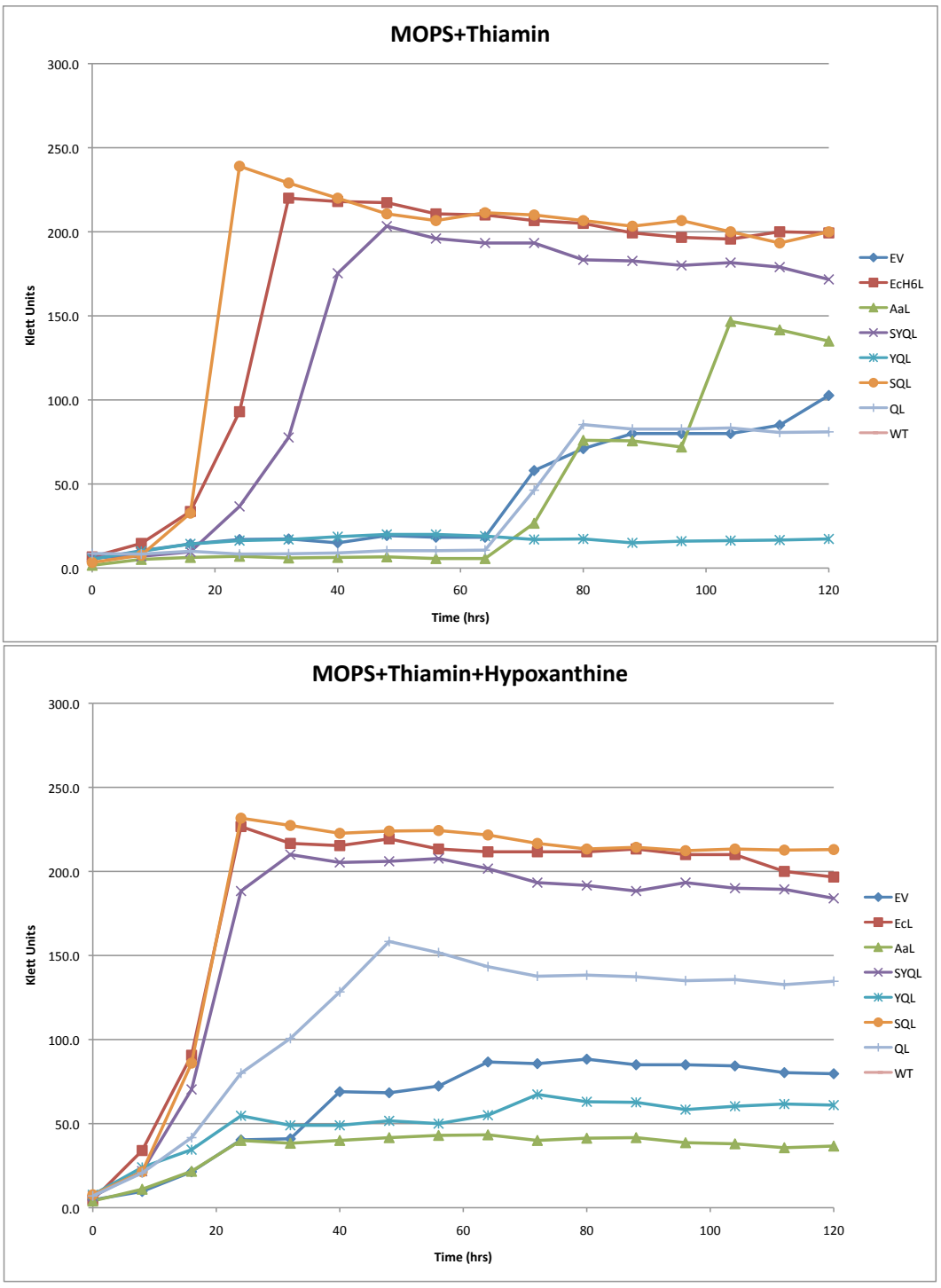

Figure 2.3 Functional complementation of AaFS proteins in JW2541 Kan^{s} in MOPS minimal medium excluding error bars.

Biological replicates were n = 3. Constructs used were pET23a (EV), pJK556 (EcH6L); pJK539 (AaL); pJK598 (SYQL); pJK605 (SQL); pJK610 (QL); and pJK623 (YQL).

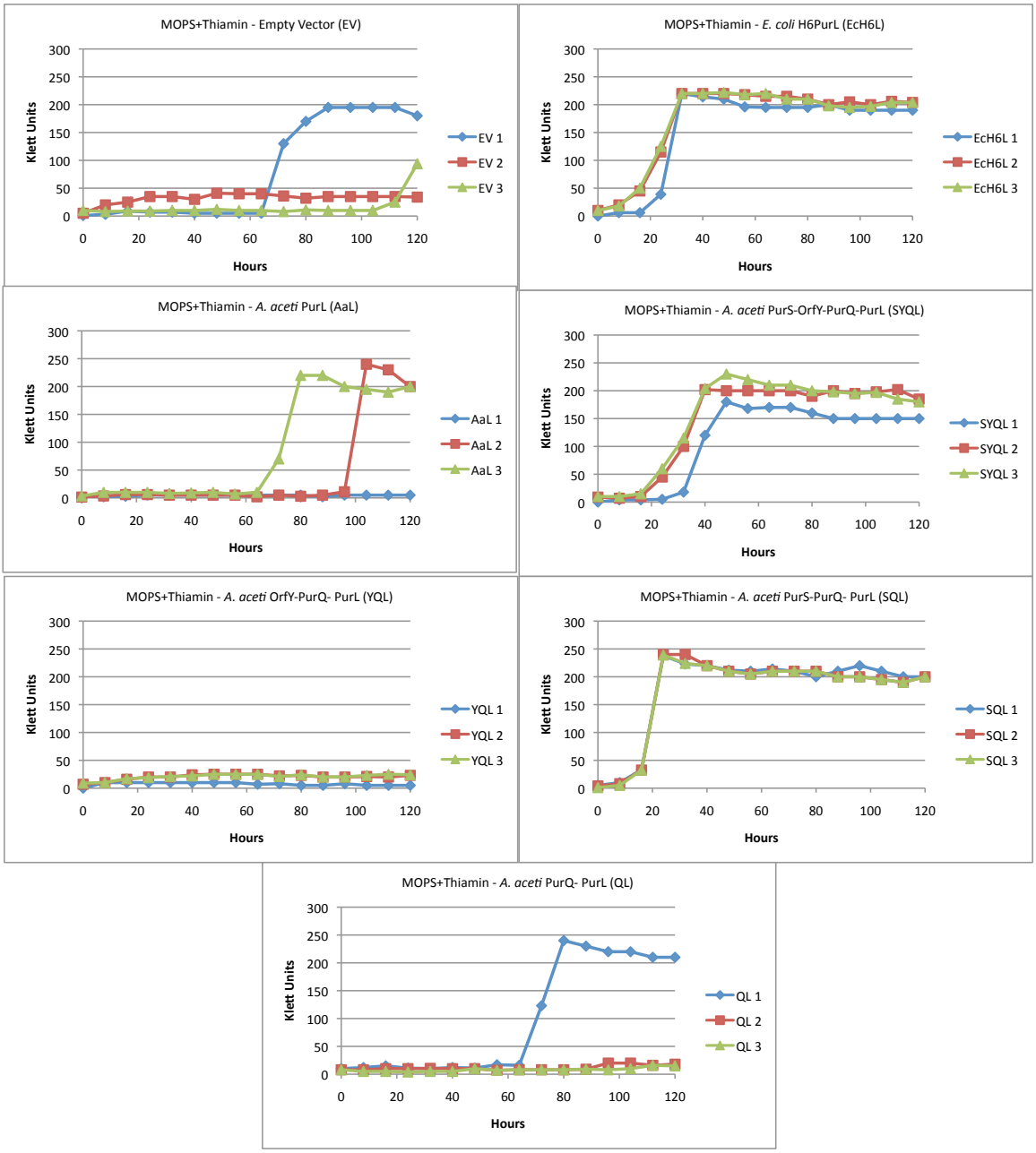

Figure 2.4 Unaveraged curves for JW2541 Kan^{s} in MOPS minimal medium with thiamin. Constructs used were pET23a (EV), pJK556 (EcH6L); pJK539 (AaL); pJK598 (SYQL); pJK605 (SQL); pJK610 (QL); and pJK623 (YQL).

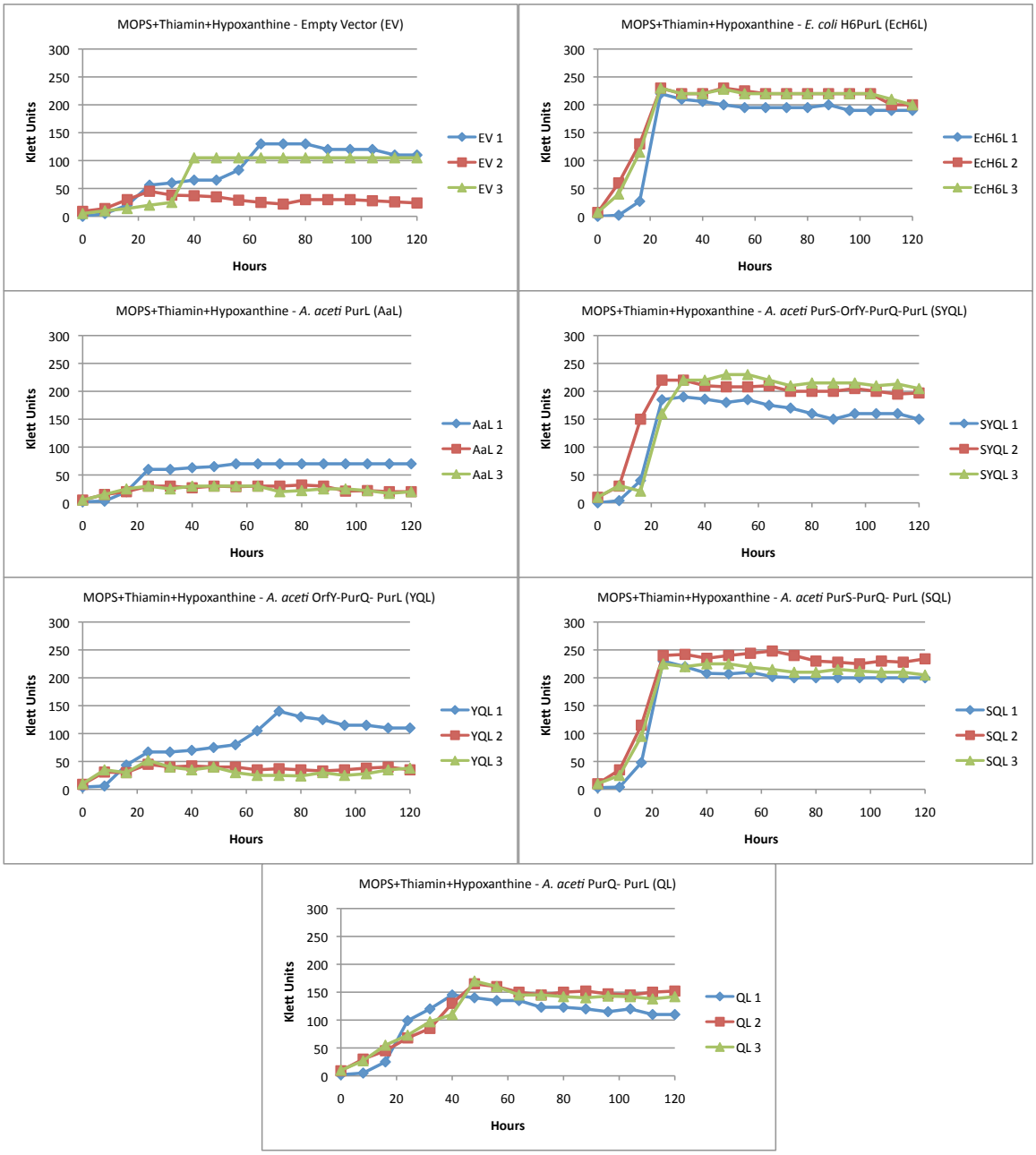

Figure 2.5 Unaveraged curves for JW2541 Kan^{s} in MOPS minimal medium with thiamin and hypoxanthine.

Constructs used were pET23a (EV), pJK556 (EcH6L); pJK539 (AaL); pJK598 (SYQL); pJK605 (SQL); pJK610 (QL); and pJK623 (YQL).

As previously observed, SQL grew similarly if not better than EcH6L. SYQL showed a slight lag in growth when compared to SQL and EcH6PurL. The QL construct showed a

significant delay in the lag phase and the EV curve surprisingly mirrored the QL growth profile, where growth was not expected. Upon disposal, cultures smelled sweet therefore growth observed after 72 hrs may not have been solely *E. coli* JW2541. Cells containing YQL did not grow much at all and an unpredictable growth pattern was observed for AaL (one culture did not grow at all, the second started at 72 hrs, and the third at 96 hrs).

A higher concentration of hypoxanthine may have to be supplemented as cultures containing nonfunctional AaFS constructs (*i.e.* AaL, YQL, and EV) could not grow past a Klett reading of 100 ($O.D._{600\ nm}$ equivalents still have not been determined experimentally - the Klett meter manual suggests the use of R = 1000 x D / 2 for conversion, where R: Klett reading; D: optical density, so 100 Klett units would correspond to 0.2 O.D. according to the formula). The other strains thrived as they carried the right combination of AaFS genes to make up active AaFS.

Use of the green filter (#54, spectral range 500-570 nm) rather than the blue filter (#42, spectral range 400-465 nm) on the Klett meter gave lower readings likely due to the absorbance of compounds (*i.e.* flavins) in the blue filter range. When the Klett meters were first obtained from Vic Rodwell, no other filters other than the blue one were available.

96-well plate MOPS complementation. The 8-hr interval readings used previously were too far apart to monitor the lag phase. Nina Serratore (Briggs laboratory) had been using a heated microplate reader for growth curves so experimental parameters were designed according to her experience. 150 uL of MOPS minimal medium were inoculated at a starting $O.D._{600\ nm}$ and monitored every two hours (n = 3). Figures 2.6 through 2.8 show the averaged curves with and without error bars and the unaveraged curves, respectively.

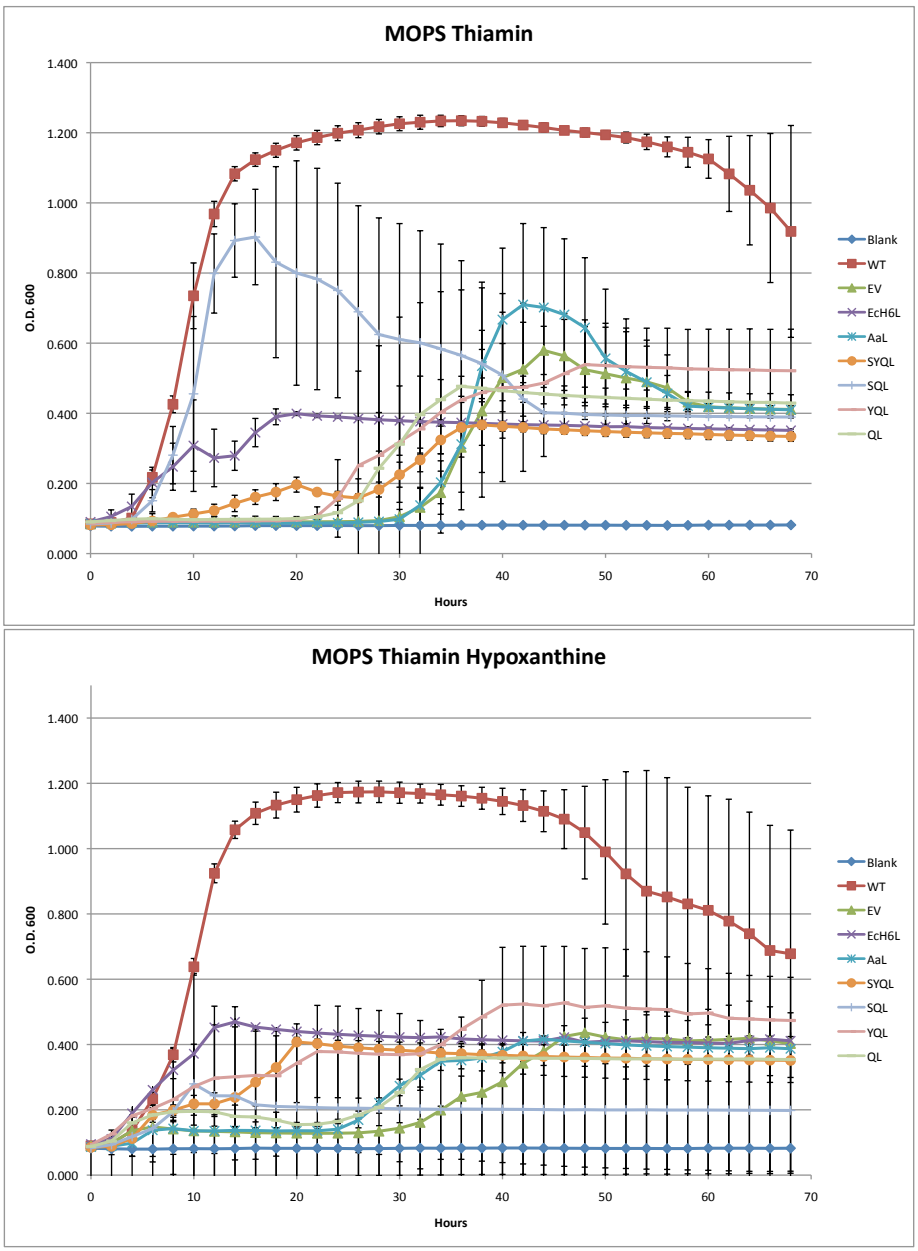

Figure 2.6 Averaged curves for the 96-well plate complementation with error bars for BW25113 (WT) and JW2541 Kan^{s} in MOPS + thiamin.

Constructs were pET23a (EV), pJK556 (EcH6L); pJK539 (AaL); pJK598 (SYQL); pJK605 (SQL); pJK610 (QL); and pJK623 (YQL).

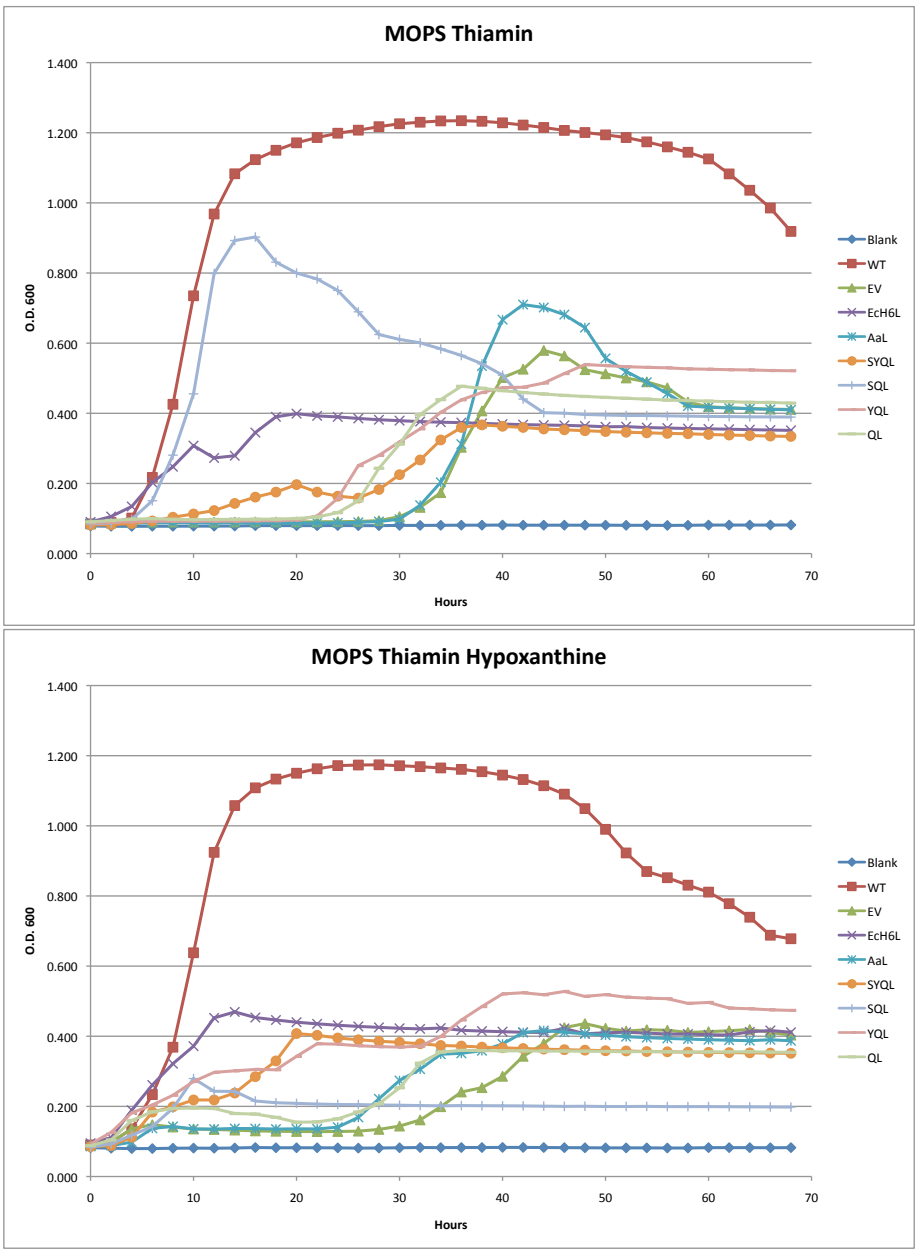

Figure 2.7 Averaged curves for the 96-well plate complementation without error bars for BW25113 (WT) and JW2541 Kan^{s} in MOPS + thiamin.

Constructs were pET23a (EV), pJK556 (EcH6L); pJK539 (AaL); pJK598 (SYQL); pJK605 (SQL); pJK610 (QL); and pJK623 (YQL).

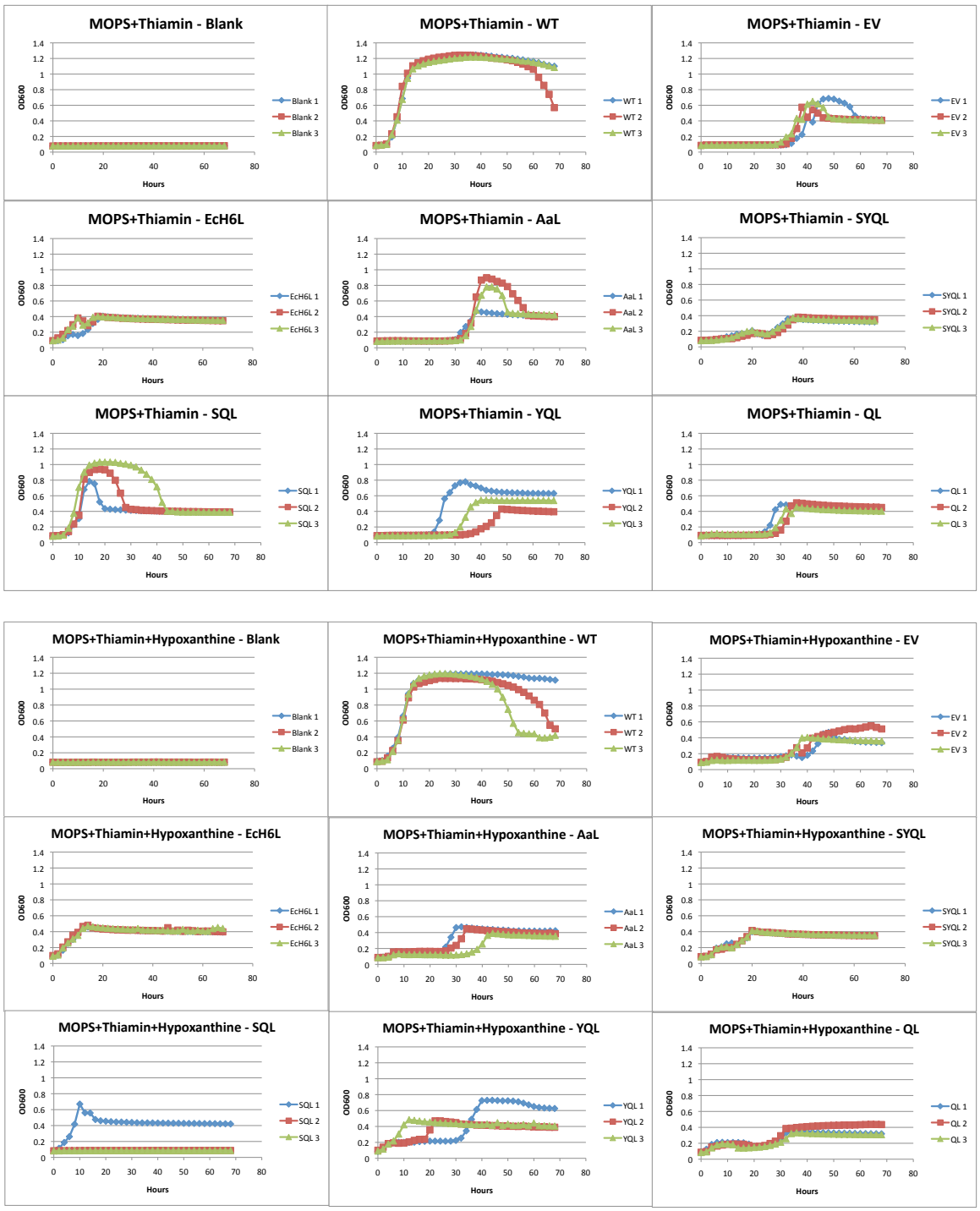

Figure 2.8 Unaveraged curves for the 96-well plate complementation for BW25113 (WT) and JW2541 Kan^{s} in MOPS + thiamin or MOPS + thiamin + hypoxanthine.

Constructs were pET23a (EV), pJK556 (EcH6L); pJK539 (AaL); pJK598 (SYQL); pJK605 (SQL); pJK610 (QL); and pJK623 (YQL).

The WT strain (BW25113) grew the best out of all of the strains tested and reached a maximum $O.D._{600\ nm}$ of ~ 1.2 (Figures 2.6-2.8). No other strain was able to reach that high of an O.D. thus indicating that the leakiness of the pET constructs was not enough to complement the *purL* deletion. JW2541 may have to have the genes of interest inserted into the genome for full complementation of the *purL* deletion.

No contamination was observed in the blanks over the course of 70 hrs (Figure 8, ± hypoxanthine). Some of the curves suddenly dipped in the middle of the growth (*i.e.* MOPS+Thiamin EcH6L), showed biphasic growth (*i.e.* MOPS+Thiamin SYQL and MOPS+Thiamin+Hypoxanthine QL), or suddenly dropped and stabilized (*i.e.* MOPS+Thiamin EV, AaL, SQL) (Figure 2.8). Nina Serratore did not observe any anomalies in her yeast growth curves but Luyen Nguyen and Michael Melesse mentioned some inconsistencies in theirs (*i.e.* the instrument appeared to reset itself in the middle of a run for random wells and at random times). Although, this experiment should be repeated when the instrument issues are fixed, the same general trends observed in the 100 mL scale were seen in the 96-well plate format (Figures 2.6 and 2.7): SQL entered the lag phase sooner than EcH6L and the latter grew better than SYQL. A delay in lag phase was observed for EV, AaL, and QL (Figures 2.6-2.8). Growth of the YQL strain was not observed in the 100 mL scale however, in the 150 uL scale, a variable delay in entering the lag phase was observed at 20, 30, 40 hrs for no hypoxanthine and 0, 20, 30 hrs for + hypoxanthine (Figure 2.8, ± hypoxanthine). No growth was observed in two out of the three hypoxanthine SQL replicates even though those wells were inoculated. It is still unclear why cells cannot grow to as high of a density as the wild type in the presence of hypoxanthine.

The 150 uL 96-well plate scale with starting $O.D._{600\ nm}$ of 0.005 seemed to work well for monitoring the functional complementation of AaFS genes in JW2541. All unused wells should be filled with water or fresh media to minimize evaporation, as recommended by Nina Serratore. Saturation of the WT strain was observed after 16 hrs and the slowest growing strain, EV, after 40 hrs (Figure 2.8), so the experiment could be done in 3 days or less.

2.4 Conclusion

OrfY is not required for AaFS activity and active AaFS is composed of PurS, PurQ, and PurL (Figure 2.4), although the stoichiometry of the subunits cannot be determined at this time. PurS does not seem necessary when PurQ and PurL are present, but there is a significant delay in growth (unless cultures were contaminated), which may indicate that similarly to TmFS, PurS may contribute to an ammonia channel that links the glutaminase and FGAR synthetase active sites [3, 4]. The *A. aceti* PurS-PurQ-PurL (SQL) construct was observed to complement as well, if not better, than *E. coli* H6PurL. Other combinations of the AaFS genes showed a significant delay in entering the lag phase. Although the wild type strain, BW25113, did not grow at the 100 mL scale (n = 1), it did grow in the 150 uL (96-well plate) format (n = 3). The same general trends for SQL, SYQL, and EcH6L were observed despite instrument issues. Surprisingly, YQL growth was observed at the 150 uL scale, but with a significant delay when compared to WT and SQL. It is still unclear why the different strains do not grow to the same density. One suggestion is to insert the genes of interest in JW2541 to test if they can complement the *purL* deletion.

The presence of OrfY was observed to cause a slight delay in the lag phase when the other three AaFS genes (PurS, PurQ, and PurL) were present, but no growth was observed in the OrfY-PurQ-PurL (YQL)-carrying strain. Taken together with the protein overexpression studies (Chapter 1), OrfY may have a regulatory role on PurQ (and potentially PurL) and be involved in iron-sulfur biosynthesis due to the proximity of *bolA* and *grx* genes (Chapter 4).

2.5 References

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CHAPTER 3. CONSTRUCTION GENERATION AND ENZYME PURIFICATION FOR FGAR SYNTHESIS

3.1 Introduction

Formylglycinamide ribonucleotide (FGAR), the FGAM synthetase (FS) substrate, cannot be chemically synthesized, therefore constructs for the *de novo* purine enzymes were obtained from *E. coli*, where they have been extensively characterized. The *de novo* purine biosynthetic pathway begins with phosphoribosylpyrophosphate (PRPP) (Figure 3.1). Since PRPP is expensive and unstable, the synthesis will start with ribose-5-phosphate (R5P).

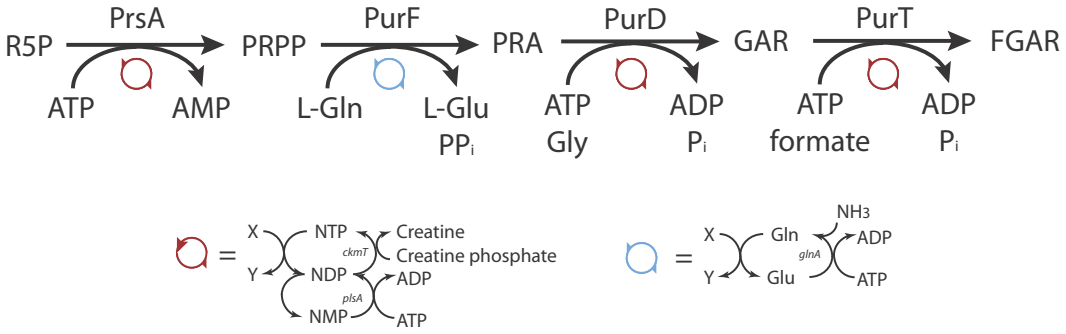

Figure 3.1 FGAR biosynthesis schema (modified from [1]).

Legend for substrates/products: R5P, ribose-5-phosphate; PRPP, phosphoribosylpyrophosphate; PRA, phosphoribosylamine; GAR, glycinamide ribonucleotide; FGAR, formylglycinamide ribonucleotide. Recycling systems are denoted by the red (ATP regeneration) and blue circles (glutamine regeneration). NTP, NDP, and NMP would have adenosine as the base.

E. coli FS will be used to monitor successful FGAR synthesis via the Bratton-Marshall assay by coupling the reaction with PurM.

3.2 Materials and Methods

Materials

All materials were from Sigma Aldrich or Fisher Scientific and of the highest purity unless otherwise noted. Vent DNA polymerase, OneTaq polymerase, all restriction endonucleases, T4 DNA ligase, and T4 polynucleotide kinase were from New England Biolabs. Phusion Hot Start polymerase was from Finnzymes. GoTaq polymerase and deoxynucleotides (dATP, dGTP, dCTP, and dTTP) were from Promega. Mutagenesis was performed using QuikChange Mutagenesis kits from Stratagene (Pfu Polymerase). Oligodeoxynucleotides (ODNs) were from Integrated DNA Technologies (Table 1). Plasmid Miniprep kits were from either Sigma, Fermentas Life Sciences, or Qiagen. QIAquick PCR Purification kit was from Qiagen. Chemically competent DH5α cells were from Invitrogen. Vectors pET23a and pET28a were from Novagen. Centrifugation steps were performed on a tabletop Eppendorf 5415D centrifuge. PCR reactions were performed in a Bio-Rad MyCycler Thermal Cycler or a MJ Research Inc. Programmable Thermal Cycler PTC-100.

Table 3.1 Oligodeoxynucleotides used in construction of plasmids and sequencing.

ODN	Sequence (5'=>3')[a]
2197	ACCTGTGGATCCCATATGTGCGGTATT
2218	CTACGCTCGAGACTTCCTTCGTTATGCATTTCGAGATTTTCC
2219	GGATATACCATGGCTAAAATCGTCGCCTGTA
2224	GTGGTGCTCGAGTGAGTTCTGCTCGCGTTCGATAG
2225	GTGGTGCTCGAGTGAGTCAAACAGGGAGGAAA
2226	GTGGTGCTCGAGTCAGTCAAACAGGGAGGAAA
2295	CACGCGTTTTCAcCGTTGGCATTGAG
2296	CTCAATGCCAACGgTGAAAACGCGTG
2332	AGTTCCCATATGTCCGCTGAACAC
2333	CTGCTTCTCGAGTTAGACGCTGTAGTA
2334	ATAGTGCTCGAGTCATCCTTCGTTATGC

[a]Letters shown in lower case code for mutagenesis.

Methods

General cloning procedure. Each recombinant gene was amplified by PCR with gene-specific primers (Table 1) from *Escherichia coli* BL21(DE3) genomic DNA, *Acetobacter*

aceti 1023 genomic DNA, or plasmid DNA as specified below. *E. coli* BL21(DE3) genomic DNA was isolated using the Promega Wizard Genomic DNA Purification kit according to the manufacturer's instructions. *A. aceti* 1023 genomic DNA was isolated using the Qiagen Genomic-tip 20/G (E. A. Mullins). *E. coli* DH5α was used for cloning and plasmid maintenance. All constructs were screened by restriction mapping or colony PCR. All numbered plasmids were verified by DNA sequencing at the Genomics Core Facility DNA Sequencing Low Throughput Laboratory at Purdue University, using primers for both directions.

Table 3.2 Plasmids used in this chapter[a].

pJK	Vector	Description	Source
515	pET23a	*EcpurTH6*[b]	TJK
556	pET22bHT	*EcH6purL*[b]	Ch. 2
592	pET23a	*EcpurD*[b]	This study
614	pET23a	*EcpurDH6*[b]	This study
621	pET28a	*EcH6purD*[b]	This study
630	pET23d	*AaprsA*[a]	This study
631	pET23d	*AaprsAH6*[a]	This study
633	pET22b	*EcH6purM*[b]	This study
638	pET23a	*EcT7purFH6*[b]	This study
639	pET23a	*EcpurFH6*[b]	This study
642	pET28a	*EcH6glnA*[b]	This study
649	pET23a	*EcpurF*[b]	This study
650	pET23a	*EcglnA*[b]	This study
668	pET22bHT	*EcH6glnA*[b]	[1]
669	pET22bHT	*EcH6plsA*[b]	[1]
670	pET22bHT	*EcH6prsA*[b]	[1]
673	pET22bHT	*EcH6purF*[b]	[1]
675	pET22bHT	*EcH6purM* G324E[b]	[1]

[a]*Aa* indicates the *A. aceti* gene. [b]*Ec* indicates the *E. coli* gene.

Construction of *E. coli* intermediate *T7*-tag*purFH6* construct plasmid pJK638. A 1.5 kb product was amplified from *E. coli* BL21(DE3) genomic DNA, using Taq Polymerase from the Chapple laboratory (Purdue University) and ODNs 2197 and 2218 with an

annealing temperature of 55°C and melting, annealing, and extension times of 20 s, 30 s, and 1 min 30 s for 25 cycles. The product was purified using a Qiagen PCR purification kit, digested with BamHI and XhoI, and ligated into the BamHI and XhoI restriction sites of the destination vector pET23a to yield pJK638. The plasmid encodes for *E. coli* T7-tagPurFH6.

Construction of *E. coli* PurFH6 construct plasmid pJK639. pJK638 was partially digested with NdeI due to an additional internal NdeI site within the *purF* sequence where the reaction was stopped after 10 min by the addition of EDTA. After using the Qiagen PCR purification kit, the digested DNA was re-ligated and digested with BamHI before transforming the reaction into DH5α cells. The plasmid, pJK639, encodes for *E. coli* PurFH6.

Construction of *E. coli purF* construct plasmid pJK649. A 1.5 kb product was amplified from pJK639, using Vent Polymerase (New England Biolabs) and ODNs 2197 and 2334 with an annealing temperature of 50°C and melting, annealing, and extension times of 30 s, 1 min, and 1 min 30 s for 25 cycles. The product was purified using a Qiagen PCR purification kit, digested with NdeI and XhoI. Digestion with NdeI was limited to 5 min where the reaction was stopped by the addition of EDTA. The digested PCR product was re-purified using a Qiagen PCR purification kit and ligated into the NdeI and XhoI restriction sites of the destination vector pET23a to yield pJK649. The plasmid encodes for *E. coli* PurF.

Construction of *E. coli purD* construct plasmid pJK592. A 1.3 kb product was amplified from BL21(DE3) genomic DNA, using ODNs 2222 and 2223 with an annealing temperature of 55°C and melting, annealing, and extension times of 20 s, 30 s, and 1min 30 s for 25 cycles using Vent polymerase. The product was purified using a Qiagen PCR purification kit, digested with NdeI and XhoI, and ligated into the NdeI and XhoI restriction sites of the destination vector pET23a to yield pJK592. The plasmid encodes for *E. coli* PurD.

Construction of *E. coli purDH6* construct plasmid pJK614. A 1.3 kb product was amplified from BL21(DE3) genomic DNA, using ODNs 2222 and 2224 with an annealing temperature of 55°C and melting, annealing, and extension times of 20 s, 30 s, and 1min 30 s for 25 cycles using Vent polymerase. The product was purified using a Qiagen PCR purification kit, digested with NdeI and XhoI, and ligated into the NdeI and XhoI restriction sites of the destination vector pET23a to yield pJK614. The plasmid encodes for *E. coli* PurDH6.

Construction of *E. coli H6purD* construct plasmid pJK621. pJK592 was digested with NdeI and XhoI and ligated into the NdeI and XhoI restriction sites of the destination vector pET28a to yield pJK621. The plasmid encodes for *E. coli* H6PurD.

Construction of *A. aceti prsA* construct plasmid pJK630. A 0.9 kb product was amplified from *A. aceti* strain 1023 genomic DNA, using ODNs 2219 and 2226 with an annealing temperature of 55°C and melting, annealing, and extension times of 20 s, 30 s, and 30 s for 25 cycles using Phusion polymerase. The product was purified using a Qiagen PCR purification kit, digested with NcoI and XhoI, and ligated into the NcoI and XhoI restriction sites of the destination vector pET23d to yield pJK630. The plasmid encodes for *A. aceti* PrsA.

Construction of *A. aceti prsAH6* construct plasmid pJK631. A 0.9 kb product was amplified from *A. aceti* strain 1023 genomic DNA, using ODNs 2219 and 2225 with an annealing temperature of 55°C and melting, annealing, and extension times of 20 s, 30 s, and 30 s for 25 cycles using Phusion polymerase. The product was purified using a Qiagen PCR purification kit, digested with NcoI and XhoI, and ligated into the NcoI and XhoI restriction sites of the destination vector pET23d to yield pJK631. The plasmid encodes for *A. aceti* PrsAH6.

Repair of *E. coli purM* construct plasmid pJK633. QuikChange mutagenesis was performed on pJK675 [1] to repair a G324E mutation using ODNs 2295 and 2296.

Construction of *E. coli H6glnA* construct plasmid pJK642. A 1.5 kb product was amplified from pJK668 using Vent Polymerase (New England Biolabs) and ODNs 2332 and 2333 with an annealing temperature of 50°C and melting, annealing, and extension times of 30 s, 1 min, and 1 min 30 s for 25 cycles. The product was purified using a Qiagen PCR purification kit, digested with NdeI and XhoI, and ligated into the NdeI and XhoI restriction sites of the destination vector pET28a to yield pJK642. The plasmid encodes for *E. coli H6GlnA* Y397F.

Construction of *E. coli glnA* construct plasmid pJK650. pJK642 was digested with NdeI and XhoI and ligated into the NdeI and XhoI sites of the destination vector pET23a to yield pJK650. The plasmid encodes for *E. coli GlnA* Y397F.

Protein expression and purification

Underlined headers indicate the final purification protocol. Antibiotics were used as follows: Amp (ampicillin, 100 ug/mL) and Kan (kanamycin, 70 ug/mL).

PurF

E. coli H6PurF (pJK673 [1]). Protocol adapted from Schultheisz et. al [1]. A single colony of BL21 (DE3) cells transformed with pJK673 [1] was used to inoculate a 50 mL LB-Amp starter culture and grown to saturation (37°C, 200 rpm). 1 L of ZYM-5052-Amp medium [2] was inoculated 1:100 and grown overnight (37°C, 200 rpm). Cells were harvested (5,000*g*, 15 min) and used immediately or stored at -80°C until use. All the following steps were performed at 4°C. The cell pellet was resuspended in 40 mL of 50 mM Tris-HCl pH 7.5, 250 mM NaCl and disrupted by sonication using a Fisher Sonic Dismembrator at 20% output (3 cycles: 1 min on, 1 min off) on ice. Cell debris was removed by centrifugation (30,000*g*, 30 min). Streptomycin sulfate (10% w/v stock) was added to a final concentration of 1% w/v, incubated on ice for 10 min, then spun (30,000*g*, 10 min). The cleared lysate was loaded onto a nickel-charged and equilibrated (in 50 mM Tris-HCl pH 7.5, 250 mM NaCl) Ni-NTA agarose column (1.5 cm x 1.5 cm, 2.7 mL) and washed with 10x column volumes of the above buffer with 20 mM

imidazole. The protein was eluted using 250 mM imidazole in the above buffer. Since the protein is catalytically inactive, it was discarded.

E. coli PurFH6 (pJK639). BL21(DE3) cells transformed with pJK639 were used to inoculate a 50 mL LB-Amp starter culture and grown to saturation (37°C, 200 rpm). 1 L of ZYM-5052-Amp [2] was inoculated 1:100 from a starter culture and grown overnight (37°C, 200 rpm). The following steps were performed at 4°C. Cells were harvested (5,000g, 15 min) and used immediately or stored at -80°. All the following steps were performed at 4°C. The cell pellet was resuspended in 50 mM phosphate buffer pH 7.5 (5 mL/g pellet) and disrupted by sonication (3 cycles, 1 min on, 1 min off) at 20-25% output using a Fisher Sonic Dismembrator. Cell debris was removed by centrifugation (30,000*g*, 30 min). Streptomycin sulfate (10% w/v stock) was added to a final concentration of 1% and the lysate was cleared by centrifugation (30,000g for 10 min). The cleared lysate was loaded onto a previously equilibrated (in 50 mM phosphate buffer pH 7.5) and charged Ni-NTA column (1.5 cm x 4.4 cm, 7.8 mL) and the column was washed with 10 column volumes of 10 mM imidazole in 50 mM phosphate buffer pH 7.5. A linear imidazole gradient (10-500 mM in 50 mM phosphate buffer pH 7.5) was used to elute the protein (20 mL x 20 mL). Fractions containing pure protein by SDS-PAGE were pooled and the protein was concentrated to 19.1 mg/mL using an an Amicon ultrafiltration 8050 cell with a Millipore PL-30 membrane. Isolated PurFH6 was stored at -80°C.

E. coli PurF (pJK649). Protocol adapted from [3, 4]. In short, BL21(DE3) cells transformed with pJK649 were used to inoculate a 50 mL LB-Amp starter culture and grown to saturation (37°C, 200 rpm). 2 L of LB-Amp were inoculated 1:100 from a starter culture and grown overnight (37°C, 200 rpm). The following steps were performed at 4°C. Cells were harvested (5,000g, 15 min) and used immediately or stored at -80°. All the following steps were performed at 4°C. The cell pellet was resuspended in Buffer A (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 5 mM MgCl2, and 1 mM glutamine (3.5 mL/g pellet)) and disrupted by sonication (3 cycles, 1 min on, 1 min off) at 20-25% output using a Fisher Sonic Dismembrator. Cell debris was removed by centrifugation (30,000*g*, 30 min). Streptomycin sulfate (10% w/v stock) was added to a final

concentration of 1% and the lysate was cleared by centrifugation (30,000g for 10 min). The cleared lysate was loaded onto a previously equilibrated (in Buffer A) DEAE-Sepharose CL6B column (2.5 x 14.5 cm, 71.2 mL) and the column was washed with 250 mL Buffer A. A linear ammonium sulfate gradient (0.14-0.3 M in Buffer A) was used to elute the protein (200 mL x 200 mL). Fractions containing activity were pooled and 28.3 mg protein was concentrated to 10.1 mg/mL using an an Amicon ultrafiltration 8050 cell with a Millipore PL-30 membrane in 57.5 mM Tris-HCl pH 7.5. Glycerol was added to a final concentration of 15%. Isolated *E. coli* PurF was stored at -80°C.

E. coli PurD.

E. coli PurD (pJK592) Prep 1. Protocol adapted from [5]. BL21(DE3) cells transformed with pJK592 were used to inoculate a 50 mL LB-Amp starter culture and grown to saturation (37°C, 200 rpm). 1 L of ZYM-5052-Amp medium [2] was inoculated 1:100 and grown overnight (37°C, 200 rpm). Cells were harvested (5,000g, 15 min) and used immediately or stored at -80°. All the following steps were performed at 4°C. The cell pellet was resuspended in 50 mM KP_i pH 7.5, 5 mM β-mercaptoethanol (BME), and 1 mM phenylmethanesulfonyl fluoride (PMSF) (5 mL/g pellet) and disrupted by sonication (3 cycles, 1 min on, 1 min off) at 20-25% output using a Fisher Sonic Dismembrator. Cell debris was removed by centrifugation (30,000*g*, 30 min). Streptomycin sulfate (10% w/v stock) was added to a final concentration of 1% and the lysate was cleared by centrifugation (30,000g for 10 min). Ammonium sulfate fractionation was performed as described previously: 0-33% (196 g/L); 33-65% (214 g/L). The 33-65% ammonium sulfate pellet was resuspended in a minimal amount of 50 mM KP_i pH 7.5, 5 mM BME, 1 mM PMSF buffer and loaded onto a Sephadex G25 medium column (2.5 cm x 34 cm, 167 mL). The column was developed and fractions containing protein ($A_{280nm} > 0.2$) were pooled and loaded onto a Whatman DE52 cellulose column (2.5 cm x 29.5 cm, 145 mL). The DE52 column was washed with 300 mL 50 mM KP_i pH 7.5, 5 mM BME, 1 mM PMSF buffer before the protein was eluted with a linear gradient (0-300 mM KCl, (300 mL x 300 mL) in 50 mM KP_i pH 7.5, 5 mM BME, 1 mM PMSF). Fractions were pooled by purity by SDS-PAGE. The protein was concentrated to 6.7 mg/mL and buffer

exchanged using an Amicon ultrafiltration 8400 cell and a YM-10 membrane. The isolated protein was discarded due to lack of detectable activity.

E. coli PurD (pJK592) Prep 2. LB-IPTG was used for protein production. 1 L of LB-Amp was inoculated 1:25 with a starter culture and grown (37°C, 200 rpm) until an $O.D._{600}$ ~0.7 was reached. IPTG was then added to a final concentration of 0.4 mM from a 1M stock. The culture was induced for 2.5 hrs at 37°C, and the cells were harvested. The same protocol as prep 1 was followed except for buffers did not contain PMSF or BME.

E. coli PurDH6 (pJK614). BL21(DE3) cells transformed with pJK614 were used to inoculate a 50 mL LB-Amp starter culture and grown to saturation (37°C, 200 rpm). 2 L of LB-Amp were inoculated 1:100 from a starter culture and grown overnight (37°C, 200 rpm). The following steps were performed at 4°C. Cells were harvested (5,000g, 15 min) and used immediately or stored at -80°C. All the following steps were performed at 4°C. The cell pellet was resuspended in 50 mM phosphate buffer pH 7.5 (3.5 mL/g pellet) and disrupted by sonication (3 cycles, 1 min on, 1 min off) at 20-25% output using a Fisher Sonic Dismembrator. Cell debris was removed by centrifugation (30,000*g*, 30 min). Streptomycin sulfate (10% w/v stock) was added to a final concentration of 1% and the lysate was cleared by centrifugation (30,000g for 10 min). The cleared lysate was loaded onto a previously equilibrated (in 50 mM phosphate buffer pH 7.5) and charged Ni-NTA column (1.5 cm x 4.9 cm, 8.7 mL) and the column was washed with 10 column volumes of 10 mM imidazole in 50 mM phosphate buffer pH 7.5. A linear imidazole gradient (10-500 mM) was used to elute the protein (30 mL x 30 mL). Fractions containing pure protein by SDS-PAGE were pooled and the protein was concentrated to 107 mg/mL using an an Amicon ultrafiltration 8050 cell with a Millipore YM-10 membrane. Isolated *E. coli* PurDH6 was discarded due to low activity.

E. coli H6PurD (pJK621) Prep 1. BL21(DE3) cells transformed with pJK621 were used to inoculate a 50 mL LB-Kan starter culture and grown to saturation (37°C, 200 rpm). 1 L of LB-Kan were inoculated 1:100 from a starter culture and grown overnight (37°C,

200 rpm). The following steps were performed at 4°C. Cells were harvested (5,000g, 15 min) and used immediately or stored at -80°. All the following steps were performed at 4°C. The cell pellet was resuspended in 50 mM phosphate buffer pH 7.5 (5 mL/g pellet) and disrupted by sonication (3 cycles, 1 min on, 1 min off) at 20-25% output using a Fisher Sonic Dismembrator. Cell debris was removed by centrifugation (30,000*g*, 30 min). Streptomycin sulfate (10% w/v stock) was added to a final concentration of 1% and the lysate was cleared by centrifugation (30,000g for 10 min). The cleared lysate was loaded onto a previously equilibrated (in 50 mM phosphate buffer pH 7.5) and charged Ni-NTA column (1.5 cm x 4.9 cm, 8.7 mL) and the column was washed with 10 column volumes of 10 mM imidazole in 50 mM phosphate buffer pH 7.5 buffer. A linear imidazole gradient (10-500 mM in 50 mM phosphate buffer pH 7.5) was used to elute the protein (25 mL x 25 mL). Fractions containing pure protein by SDS-PAGE were pooled and the protein (14.4 mg) was concentrated to 2.9 mg/mL using an an Amicon ultrafiltration 8050 cell with a Millipore PL-30 membrane. Isolated *E. coli* H6PurD was not kept.

E. coli H6PurD (pJK621) Prep 2. Same protocol as prep 1 except 1 L ZYM-5052-Kan [2] was used as the production culture and 1 mM PMSF was added to the buffers. The isolated protein (117 mg) was concentrated to 14.7 mg/mL and stored at -80°C.

E. coli PurTH6 (pJK515). A C41(DE3) cell pellet grown by T. J. Kappock was resuspended in 50 mM Tris-HCl pH 8.0 (5 mL/g cell pellet) and disrupted by sonication using a Fisher Sonic Dismembrator at 20% output (3 cycles: 1 min on, 1 min off) on ice. All the following steps were performed at 4°C. Cell debris was removed by centrifugation (30,000*g*, 30 min). Streptomycin sulfate (10% w/v stock) was added to a final concentration of 1% w/v, incubated on ice for 10 min, then spun (30,000*g*, 10 min). Ammonium sulfate fractionation was performed by the addition of solid ammonium sulfate over the course of 30 min and letting the solution equilibrate for another 30 min; 0-25% (144 g/L) and 25-45% (123 g/L). Precipitated protein was cleared by centrifugation (30,000*g*, 10 min). The 25-45% pellet was resuspended in a minimal amount of 50 mM Tris-HCl pH 8.0 and loaded onto a nickel-charged and equilibrated (in

50 mM Tris-HCl pH 8.0) Ni-NTA agarose column (1.75 cm x 2.2 cm, 5.3 mL) and washed with 10x column volumes of 10 mM imidazole in 50 mM Tris-HCl pH 8.0. The protein was eluted using a linear imidazole gradient (10-500 mM in 50 mM Tris-HCl pH 8.0, 30 mL x 30 mL). Fractions containing pure protein by SDS-PAGE were pooled, concentrated to 36.2 mg/mL (145 mg total), and buffer exchanged into 50 mM Tris-HCl pH 8.0 using an Amicon Ultra-15 30,000 MWCO centrifugal device (4,000 rpm, 15 min). Isolated *E. coli* PurTH6 was stored at -80°C.

E. coli H6PlsA (pJK669) [1]. Protocol adapted from Schultheisz et. al [1]. A single colony of BL21 (DE3) cells transformed with pJK669 [1] was used to inoculate a 50 mL LB-Amp starter culture and grown to saturation (37°C, 200 rpm). 1 L of ZYM-5052-Amp medium [2] was inoculated 1:100 and grown overnight (37°C, 200 rpm). Cells were harvested (5,000*g*, 15 min) and used immediately or stored at -80°C until use. All the following steps were performed at 4°C. The cell pellet was resuspended in 40 mL of 50 mM Tris-HCl pH 7.5, 250 mM NaCl and disrupted by sonication using a Fisher Sonic Dismembrator at 20% output (3 cycles: 1 min on, 1 min off) on ice. Cell debris was removed by centrifugation (30,000*g*, 30 min). Streptomycin sulfate (10% w/v stock) was added to a final concentration of 1% w/v, incubated on ice for 10 min, then spun (30,000*g*, 10 min). The cleared lysate was loaded onto a nickel-charged and equilibrated (in 50 mM Tris-HCl pH 7.5, 250 mM NaCl) Ni-NTA agarose column (1.5 cm x 1.5 cm, 2.7 mL) and washed with 10x column volumes of the above buffer with 20 mM imidazole. The protein was eluted using 250 mM imidazole in the above buffer. Fractions containing pure protein by SDS-PAGE were pooled, concentrated to 7.5 mg/mL (37 mg protein total), and buffer exchanged into 50 mM postassium phosphate, 5 mM β-ME, 50% glycerol using an an Amicon ultrafiltration 8050 cell with a Millipore PL-5 membrane. Isolated *E. coli* H6PlsA was stored at -20°C.

PrsA

E. coli H6PrsA (pJK670 [1]) prep 1. Same protocol as for the isolation of *E. coli* H6PlsA above, using a JM109(DE3) cell pellet grown by T. J. Kappock. The Ni-NTA column was 1.5 cm x 1.5 cm, 2.7 mL. The protein was discarded due to low yield.

E. coli H6PrsA (pJK670 [1]) Prep 2. Same protocol as prep 1 but using BL21(DE3) cells grown in ZYM-5052-Amp [2]. A Millipore YM-10 was used in the Amicon ultrafiltration 8050 cell. The protein was discarded due to low yield.

E. coli H6PrsA (pJK670 [1]) Prep 3. Protocol adapted from [6]. In short, the BL21(DE3) cell pellet (ZYM-5052-Amp [2]) was resuspended in 35 mL 50 mM KP_i, pH 6.9 and disrupted by sonication using a Fisher Sonic Dismembrator at 20-25% output (3 cycles: 1 min on, 1 min off) on ice. Cell debris was removed by centrifugation (30,000*g*, 30 min). Streptomycin sulfate (10% w/v stock) was added to a final concentration of 1% w/v, incubated on ice for 10 min, then spun (30,000*g*, 10 min). The soluble lysate was heated to 55°C as follows. The beaker containing lysate was placed in a boiling water bath until the temperature reached 54°C and transferred to a 55°C water bath for 5 min. The solution was transferred into an ice cold beaker and chilled for 5 min. Denatured proteins were removed by centrifugation (13,000*g*, 20 min). Ammonium sulfate fractionation was performed as described previously: 0-35% (209 g/L). After centrifugation, the pellet was resuspended in a minimal amount of 50 mM KP_i, pH 6.9 buffer. The protein was discarded due to low yield.

E. coli H6PrsA (pJK670 [1]) Prep 4. Protocol adapted from [7]. In short, the BL21(DE3) cell pellet (ZYM-5052-Amp [2]) was resuspended in 50 mM NaH_2PO_4 pH 8.0, 1 M NaCl (5 mL/g pellet) and disrupted by sonication using a Fisher Sonic Dismembrator at 20-25% output (3 cycles: 1 min on, 1 min off) on ice. Cell debris was removed by centrifugation (30,000*g*, 30 min). The soluble lysate was loaded onto a nickel-charged and equilibrated Ni-NTA agarose column (in 50 mM NaH_2PO_4 pH 8.0, 1 M NaCl, 1.5 cm x 1.5 cm, 2.7 mL) and washed with 10x column volumes of the above buffer with 75 mM imidazole. The protein was eluted using 75 mM imidazole in the above buffer. Fractions containing pure protein by SDS-PAGE were pooled, concentrated to 0.2 mg/mL, and buffer exchanged into 50 mM NaH_2PO_4 pH 8.0, 400 mM NaCl using an an Amicon ultrafiltration 8050 cell with a Millipore YM-10 membrane. The protein was discarded due to low yield.

E. coli H6PrsA (pJK670 [1]) Prep 5. Protocol adapted from [8]. The BL21(DE3) cell pellet (ZYM-5052-Amp [2]) was resuspended in 50 mM KPi, pH 7.5 (5 mL/g cell pellet) and disrupted by sonication using a Fisher Sonic Dismembrator at 20-25% output (3 cycles, 1 min on, 1 min off). All the following steps were performed at 4°C. Cell debris was removed by centrifugation (30,000*g*, 30 min). Streptomycin sulfate (10% w/v stock) was added to a final concentration of 1% and the lysate was cleared by centrifugation (30,000*g*,10 min). The streptomycin sulfate pellet was resuspended in a minimal amount of 50 mM KPi, pH 7.5 and dialyzed overnight against 200 volumes (1 L) of 50 mM KPi, pH 7.5 (no changes). The dialyzed extract was cleared by centrifugation as above and loaded onto a previously equilibrated (in 50 mM KPi, pH 7.5) and charged Ni-NTA column (4.8 cm x 1.5 cm, 8.5 mL). The column was washed with 10 column volumes of 10 mM imidazole in 50 mM KPi, pH 7.5 buffer and the protein was eluted using a linear 10-500 mM imidazole gradient in 50 mM KPi, pH 7.5 (25 mL x 25 mL, 200 drop fractions). Ammonium sulfate precipitation (0-25%, 144 g/L) was performed on the flowthrough of the column. The protein was stored as an ammonium sulfate 75% (516 g/L) slurry at 4°C.

A. aceti PrsAH6 (pJK631). pJK631 was expressed in BL21(DE3) using ZYM-5052 (37°C) [2], C41(DE3) using LB-IPTG, and C41(DE3)/pREP4-GroESL using LB-IPTG as described above.

GlnA

E. coli H6GlnA Y397F (pJK642) Prep 1. BL21(DE3) cells transformed with pJK642 were used to inoculate a 50 mL LB-Kan starter culture and grown to saturation (37°C, 200 rpm). 1 L of ZYM-5052-Kan [2] was inoculated 1:100 from a starter culture and grown overnight (37°C, 200 rpm). The following steps were performed at 4°C. Cells were harvested (5,000g, 15 min) and used immediately or stored at -80°. All the following steps were performed at 4°C. The cell pellet was resuspended in 50 mM Tris-HCl pH 8.0, 100 mM KCl (5 mL/g pellet) and disrupted by sonication (3 cycles, 1 min on, 1 min off) at 20-25% output using a Fisher Sonic Dismembrator. Cell debris was removed by centrifugation (30,000*g*, 30 min). Streptomycin sulfate (10% w/v stock) was

added to a final concentration of 1% and the lysate was cleared by centrifugation (30,000g for 10 min). The cleared lysate was loaded onto a previously equilibrated (in 50 mM Tris-HCl pH 8.0, 100 mM KCl) and charged Ni-NTA column (1.5 x 1.5 cm, 2.7 mL) and the column was washed with 10 column volumes of 10 mM imidazole buffer in 50 mM Tris-HCl pH 8.0, 100 mM KCl. A linear imidazole gradient (10-500 mM in 50 mM Tris-HCl pH 8.0, 100 mM KCl) was used to elute the protein (5 mL x 5 mL). Fractions containing pure protein by SDS-PAGE were pooled and the protein was concentrated to 2.8 mg/mL using an an Amicon ultrafiltration 8050 cell with a Millipore PL-30 membrane. Isolated *E. coli* H6GlnA Y397F was stored at -80°C.

E. coli H6GlnA Y397F (pJK642) Prep 2. Protocol adapted from [9]. In short, BL21(DE3) cells transformed with pJK642 were grown and harvested as in prep 1. The cell pellet was resuspended in 10 mM imidazole, 50 mM $MgCl_2$ pH 7.0 (3 mL/g pellet) and disrupted as in prep 1. The pH of the soluble lysate was adjusted to pH 5.85 by the addition of fresh 1 M KOH or 1 M acetic acid and monitored using a pH meter. 1 g of solid streptomycin sulfate was added for each 100 mL of extract and equilibrated with mixing on ice. The pH was again adjusted to pH 5.85 following the streptomycin sulfate equilibration with fresh 1 M KOH or 1 M acetic acid and monitored using a pH meter. The cleared supernatant (30,000*g*, 30 min) was brought to 25˚C using a water bath. $ZnSO_4$ was added to 1.5 mM [final Zn^{2+}] and the solution was incubated at 25˚C for 30 min. The precipitated protein was centrifuged (30,000*g*, 30 min) and the pellet was washed in half the original volume of cold resuspension buffer (1.5 mL/g original cell pellet) and centrifuged (30,000*g*, 30 min). The washed protein pellet was dissolved in 2.5 mM $MgCl_2$, 10 mM imidazole, pH 7.0 (1.5 mL/g original cell pellet). Insoluble material was pelleted (12,000*g*, 15 min). $MgCl_2$ was added to the supernatant to 50 mM [final $MgCl_2$] and incubated at 25˚C for 30 min with occasional stirring (gradual increase in turbidity over time). The solution was centrifuged (30,000*g*, 30 min). The pellet was dissolved in 75% of the volume of the post-12,000*g* spin in 2.5 mM $MgCl_2$, 10 mM imidazole, pH 7.0. Insoluble material was removed by centrifugation (12,000*g*, 15 min). The isolation was stopped at this point due to the lack of a visible pellet.

E. coli GlnA (pJK650). Same protocol as for prep 2 for the isolation of *E. coli* H6GlnA. Following the 25˚C Mg^{2+} crystallization, another $MgCl_2$ crystallization was repeated at 37˚C as previously described [9]. In short, the same procedure as the 25˚C crystallization was used for the 37˚C crystallization. The protein pellet was resuspended in a minimal amount of 2.5 mM $MgCl_2$, 10 mM imidazole, pH 7.0 buffer and dialyzed overnight in 200 volumes (1 L) of 10 mM imidazole, 100 mM KCl, 1 mM $MgCl_2$, pH 7.0 and stored at 4˚C as previously described [10].

E. coli H6PurL (pJK556). A single colony of BL21(DE3) transformed with pJK556 was used to inoculate a 50-mL LB-Amp starter culture and grown to saturation (37°C, 200 rpm). 1 L of ZYM-5052-Amp medium [2] was inoculated 1:100 and grown overnight (37°C, 200 rpm). Cells were harvested (5,000*g*, 15 min) and stored at -80°C until use. All the following steps were performed at 4°C. The cell pellet was resuspended in 50 mM Tris-HCl pH 8.0, 100 mM KCl (5 mL/g pellet) and disrupted by sonication using a Fisher Sonic Dismembrator at 20% output (3 cycles: 1 min on, 1 min off) on ice. Cell debris was removed by centrifugation (30,000*g*, 30 min). Streptomycin sulfate (10% w/v stock) was added to a final concentration of 1% w/v, incubated on ice for 10 min, then spun (30,000*g*, 10 min). The cleared lysate was loaded onto a previously equilibrated (50 mM Tris-HCl pH 8.0, 100 mM KCl) and charged Ni-NTA column (4.9 cm x 1.5 cm, 8.7 mL) and the column was washed with 10 column volumes of 10 mM imidazole buffer in 50 mM Tris-HCl pH 8.0, 100 mM KCl. A linear imidazole gradient (10-500 mM in 50 mM Tris-HCl pH 8.0, 100 mM KCl) was used to elute the protein (20 mL x 20 mL). Fractions containing pure protein by SDS-PAGE were pooled and the protein was concentrated to 16.0 mg/mL using an an Amicon ultrafiltration 8050 cell with a Millipore PL-30 membrane. Isolated *E. coli* H6PurL was stored at -80°C.

E. coli H6PurM (pJK633). BL21(DE3) cells transformed with pJK633 were used to inoculate a 50 mL LB-Amp starter culture and grown to saturation (37°C, 200 rpm). 1 L of ZYM-5052-Amp medium [2] was inoculated 1:100 and grown overnight (37°C, 200 rpm). Cells were harvested (5,000g, 15 min) and used immediately or stored at -80°. All the following steps were performed at 4°C. The cell pellet was resuspended in 50 mM

Tris pH 8.0, 300 mM KCl. (5 mL/g pellet) and disrupted by sonication (3 cycles, 1 min on, 1 min off) at 20-25% output using a Fisher Sonic Dismembrator. Cell debris was removed by centrifugation (30,000*g*, 30 min). Streptomycin sulfate (10% w/v stock) was added to a final concentration of 1% and the lysate was cleared by centrifugation (30,000g for 10 min). The cleared lysate was loaded onto a previously equilibrated (in 50 mM Tris pH 8.0, 300 mM KCl) and charged Ni-NTA column (1.5 cm x 4.9 cm, 8.7 mL) and the column was washed with 5 column volumes of 10 mM imidazole in 50 mM Tris pH 8.0, 300 mM KCl buffer. A linear imidazole gradient (10-500 mM in 50 mM Tris pH 8.0, 300 mM KCl) was used to elute the protein (15 mL x 15 mL). Fractions containing pure protein by SDS-PAGE were pooled and the protein was concentrated to 18.6 mg/mL (136 mg total) using an an Amicon ultrafiltration 8050 cell with a Millipore YM-10 membrane. Isolated *E. coli* H6PurM was stored at -80°C.

Activity Assays

PurF. The final assay conditions for PurF were 100 mM Tris-HCl pH 8.0, 50 mM KCl, 1 mM magnesium acetate, 2 mM glycine, 2 mM glutamine, 2 mM ATP, 2 mM PEP, 0.2 mM NADH, 15 units/mL LDH/PK mix (Sigma P0294 solution, according to the manufacturer's determined activity), 0.5 mM phosphoribosylpyrophosphate (PRPP), 5 U PurD, and PurF in a final volume of 700 uL. The reaction mix was pre-incubated at 24°C for 2 min, and the reaction was initiated by the addition of PRPP in a quartz 0.5 mL cuvette (1 cm pathlength). Enzymatic activity was monitored at 24°C in an Agilent diode-array spectrophotometer by the decrease at 340 nm with $\varepsilon = 6200$ M-1 cm-1. No detectable activity was observed prior to PRPP addition.

PurD. The final assay conditions were 100 mM Tris-HCl (pH 8.0), 50 mM KCl, 1 mM phosphoenolpyruvate (PEP), 0.2 mM NADH, 15 units/mL each of LDH/PK (Sigma P0294 solution, according to the manufacturer's determined activity), 2 mM magnesium acetate, 1 mM phosphoribosylamine (PRA), 2 mM glycine, 2 mM ATP, and PurD in a final volume of 700 uL. The reaction mix was pre-incubated at 18°C for 2 min, and the reaction was initiated by the addition of PRA in a quartz 0.5 mL cuvette (1 cm pathlength). PRA was prepared as previously described [5]. Enzymatic activity was

monitored at 18°C in an Agilent diode-array spectrophotometer or an Agilent Cary spectrophotometer by the decrease at 340 nm with ε = 6200 M-1 cm-1. No detectable activity was observed prior to PRA addition.

PurTH6. Assay conditions were adapted from the PurD assay above. The final assay conditions for PurTH6 were 100 mM Tris-HCl pH 8.0, 50 mM KCl, 2 mM magnesium acetate, 2 mM glycine, 2 mM ATP, 1 mM PEP, 0.2 mM NADH, 1 mM phosphoribosylamine (PRA), 5 units/mL LDH/PK mix (Sigma P0294 solution, according to the manufacturer's determined activity), 4 units H6PurD, 5 units H6PrsA, and PurTH6 in a final volume of 700 uL. The reaction mix was pre-incubated at 18°C for 2 min, and the reaction was initiated by the addition of PRA in a quartz 0.5 mL cuvette (1 cm pathlength). Enzymatic activity was monitored at 18°C in an Agilent diode-array spectrophotometer by the decrease at 340 nm with ε = 6200 M-1 cm-1. No detectable activity was observed prior to PRA addition. Calculated activity was divided by 2 to take into account the generation of 2 ADP molecules from the PurD and PurT reactions.

H6PlsA. The final assay conditions for H6PlsA were 100 mM Tris-HCl pH 8.0, 50 mM KCl, 2 mM magnesium acetate, 2 mM AMP, 2 mM ATP, 1 mM PEP, 0.2 mM NADH, 5 units/mL LDH/PK mix (Sigma P0294 solution, according to the manufacturer's determined activity), and H6-PlsA in a final volume of 700 uL. The reaction mix was pre-incubated at 24°C for 2 min, and the reaction was initiated by the addition of AMP in a quartz 0.5 mL cuvette (1 cm pathlength). Enzymatic activity was monitored at 24°C in an Agilent diode-array spectrophotometer by the decrease at 340 nm with ε = 6200 M-1 cm-1. No detectable activity was observed prior to AMP addition. Calculated activity was divided by 2 to take into account the generation of 2 ADP molecules per reaction.

H6PrsA. The final assay conditions for H6PrsA were 100 mM Tris-HCl pH 8.0, 50 mM KCl, 2 mM magnesium acetate, 2 mM ATP, 1 mM PEP, 0.2 mM NADH, 2 mM ribose-5-phosphate (R5P), 5 units/mL LDH/PK mix (Sigma P0294 solution, according to the manufacturer's determined activity), 5 units H6-PlsA, and H6-PrsA in a final volume of 700 uL. The reaction mix was pre-incubated at 24°C for 2 min, and the reaction was

initiated by the addition of R5P in a quartz 0.5 mL cuvette (1 cm pathlength). Enzymatic activity was monitored at 24°C in an Agilent diode-array spectrophotometer by the decrease at 340 nm with ε = 6200 M-1 cm-1. No detectable activity was observed prior to R5P addition.

GlnA. Assay conditions were adapted from the PurD assay above. The final assay conditions for GlnA were 100 mM Tris-HCl pH 8.0, 50 mM KCl, 1 mM magnesium acetate, 3 mM ATP, 2 mM PEP, 0.2 mM NADH, 15 units/mL LDH/PK mix (Sigma P0294 solution, according to the manufacturer's determined activity), 50 mM glutamate, and 40 mM ammonium chloride in a final volume of 700 uL. The reaction mix was pre-incubated at 37°C for 2 min, and the reaction was initiated by the addition of glutamate in a quartz 0.5 mL cuvette (1 cm pathlength). Enzymatic activity was monitored at 37°C in an Agilent diode-array spectrophotometer by the decrease at 340 nm with ε = 6200 M-1 cm-1. No detectable activity was observed prior to glutamate addition.

3.3 Results and Discussion

E. coli PurF. The JRW construct (pJK673 [1]) was purified (data not shown), however, previous characterization of the non-iron sulfur cluster enzyme indicated that the N-term cysteine residue is the catalytic nucleophile [11]. Therefore the protein encoded by pJK673 should code for a catalytically inactive PurF since it codes for H6PurF, so the activity was not tested. The C-term His-tagged construct was obtained (pJK639) and purified (data not shown). A total of 95 mgs of protein was isolated; however, no activity was detected, even when PRPP was used as the starting substrate. The untagged construct *E. coli* PurF (pJK649) was obtained as it was characterized previously [12] (Figure 3.2 and Table 3.3).

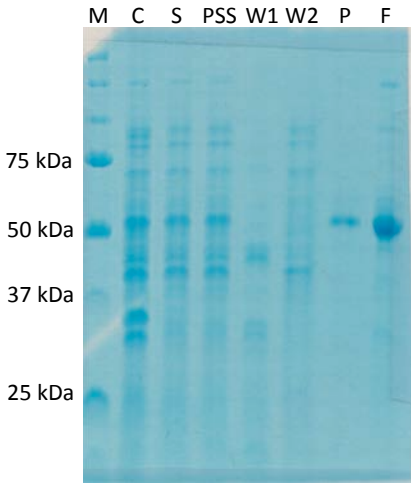

Figure 3.2 Purification of *E. coli* PurF (pJK649).

Final purification gel. SDS-PAGE (9% acrylamide), 5 ug protein/lane. Legend: C - crude; S - soluble; PSS- post streptomycin sulfate; W1 and W2 - washes 1 and 2; P - pooled elution fractions; F - pure PurF.

Table 3.3 Summary of the PurF purification.

Step	Volume (mL)	Protein (mg)	Sp. Activity (U/mg)	Activity (U)	Purification (fold)
Soluble	35	495.4	0.081	40.1	1
Strep Sulf	37.5	464.3	0.115	53.4	1.4
DEAE	90	184.32	0.244	45.0	3.0
Pure PurF	2.6	28.34	18.46	523	228

28.3 mg of *E. coli* PurF was obtained from 2 L of culture with a specific activity of 18.5 U/mg at 24°C, compared to 17.7 U/mg glutamine-dependent specific activity as previously reported at 37°C [11].

E. coli PurD (pJK592). pJK72 [1] contained silent A38 and G382 mutations and multiple sequencing attempts from the T7 promoter region failed, so the gene was cloned in-house (*purD* - pJK592; *purDH6* - pJK614; *H6purD* - pJK621). *B. subtilis PurD* (pJK034) was purified by K. Nyffeler; however the protein sat diluted for over a week at 4°C and activity was very low; since then, isolating *B. subtilis PurD* again has not been attempted. Although there were issues at the beginning with setting up the activity assay, all of the purified PurD proteins (tagged and untagged) were assayed again for activity as soon as the assay was confirmed to work with H6PurD (pJK621).

The *E. coli* PurD (pJK592) construct was shown to be leaky (data not shown); growth in ZYM-5052 failed to reproducibly induce expression of the protein. Prep 1 failed to yield active or clean protein (data not shown). Prep 2 yielded 20 mg of isolated protein (gels not shown) , with a specific activity of 3 U/mg. Future isolations of untagged PurD should include a low-salt wash (100-150 mM KCl) of the DE52 column prior to elution and the DE52 elution should be performed in a higher linear salt gradient (150 mM - 1M KCl) since PurD was found in the last 3 fractions (data not shown).

E. coli PurDH6 (pJK614). PurDH6 had the same leaky expression as the untagged version (data not shown). A total of 186 mg of protein was obtained (gels not shown) and had 0.19 U/mg activity. A doublet was observed in the purified protein lane that may correspond to proteolytic cleavage products. Since both the untagged and the C-term His-tagged proteins were not very active, the N-terminally tagged protein construct (which has been previously characterized [13]) was obtained.

E. coli H6PurD (pJK621). Prep 1 yielded 14.4 mg of protein (gels not shown) with a specific activity of 79.9 U/mg, which is about four times the specific activity that was reported previously [13]. Error in calculations and the Bradford may be attributed to the large difference in specific activity. A doublet was again observed in the purified protein lane so PMSF may be a necessary addition to the buffers. Since not much protein was obtained, the purification was attempted once more.

Prep 2. Cells were grown in ZYM-5052 [2] to increase protein yield. The purification gels are shown in Figure 3.3.

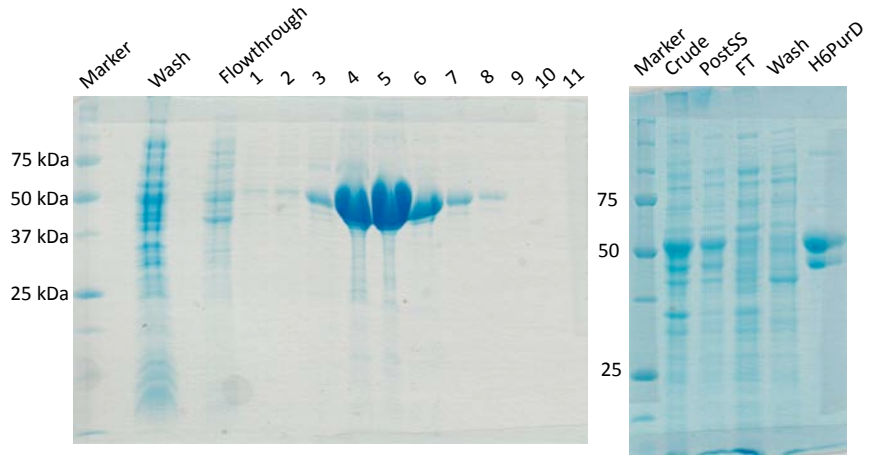

Figure 3.3 Purification of H6PurD (pJK621) prep 2.

Left, elution gel, 10 uL/lane (protein amount unknown). Numbers denote elution fractions. Right, final purification gel, 5 ug protein/lane. SDS-PAGE (9% acrylamide).

A total of 117 mg of protein was isolated with a specific activity of 40 U/mg, which is similar to the previously characterized wild-type *E. coli* PurD [14] but twice of that of H6PurD [13]. Even though PMSF was present in the buffers, the lower molecular weight band, likely due to proteolysis was still present. Active PurD has finally been isolated!

E. coli PurTH6 (pJK515). PurT, or GAR tranformylase, catalyzes the third step in the *de novo* purine pathway and the C-terminally His-tagged version was isolated as shown in Figure 3.4.

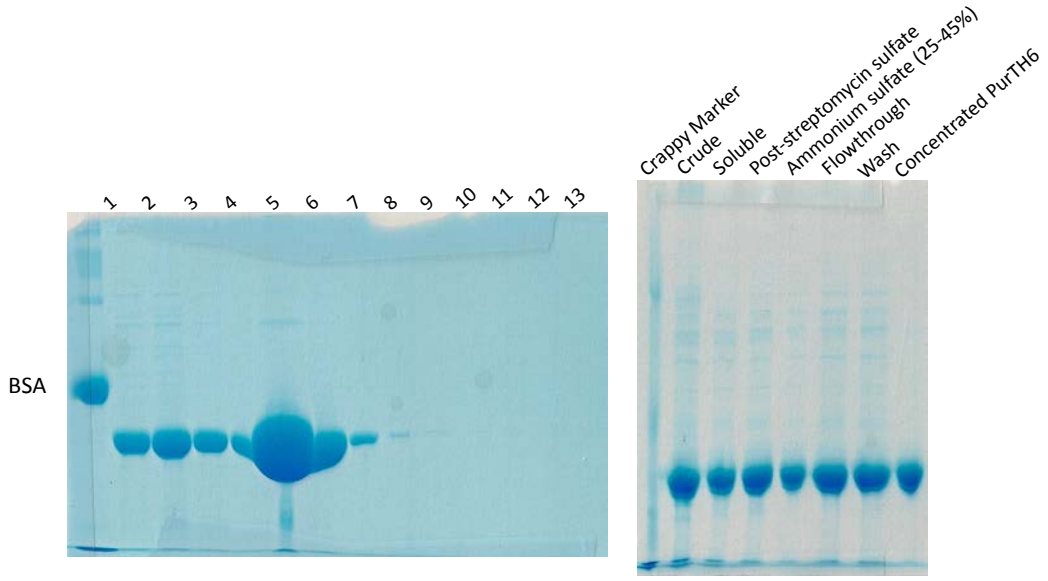

Figure 3.4 Purification of PurTH6 (pJK515).

Left, elution profile, 10 uL/lane (protein amount unknown). Numbered lanes denote the elution fractions. Right, final purification gel, 5 ug/protein/lane. SDS-PAGE (9% acrylamide).

The column was overloaded as PurTH6 was present in the wash; however 145 mg of protein was isolated (fractions 2-6 were pooled, Figure 3.4). When formate was omitted from the activity assay, the total ATPase activity was calculated to be 0.25 U. Once formate was added, the total ATPase activity increased to 0.29 U (previous calculation of 1.5 U/mg value was wrong). The C-term form of the enzyme has not been characterized previously; however, the untagged *E. coli* protein was reported to have a specific activity of 52 ug/min*mg*mL [15]. It is important to note that the enzymatic activity of PurTH6 was tested over a year after purification and no enzyme dilutions were made. The C-term His-tag may be to blame as it may interfere with homodimer formation as it is located close to the dimer interface (PDB 1KJ8). H6PurT (not previously characterized) may yield active protein as a maltose-binding protein (MBP) N-term tag PurT was characterized previously [16].

E. coli H6PlsA (pJK669 [1]). PlsA, or adenylate kinase, catalyzes the γ-phosphate transfer from ATP to AMP to yield two ADPs. The elution and purification gels are shown in Figure 3.5.

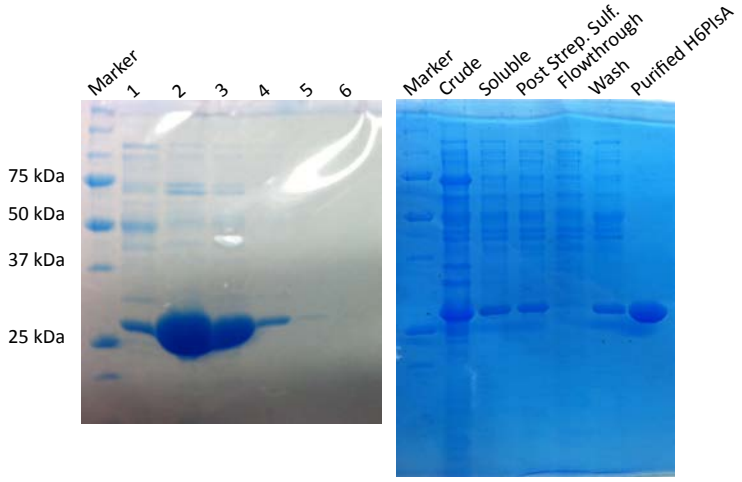

Figure 3.5 Purification of H6PlsA.

Left, elution gel, 10 uL/lane (protein amount unknown). Numbered lanes denote the elution fractions. Right, final purification gel, 5 ug/lane. SDS-PAGE (12% acrylamide).

37 mg of protein was isolated with a specific activity of 1.9 U/mg for the reaction, or 0.9 U/mg/ADP; compared to 1 U for the N-term tagged-protein [1] (taking into account of the ADP molecules in activity calculation was not mentioned).

PrsA. PrsA, or PRPP synthase, catalyzes the phosphorylation of ribose-5-phosphate to yield PRPP.

E. coli H6PrsA (pJK670 [1]). Sequencing of this construct did not reveal any mutations. Expression in JM109(DE3) cells showed no induction (prep 1, data not shown) so the construct was isolated from BL21(DE3) cells in prep 2, where 1 mg was isolated with a specific activity of 0.9 U/mg, compared to 2.5 U/mg as previously reported for the C-term tagged form [1]. The protein was observed to precipitate out after streptomycin sulfate treatment in preps 2 and 3 (data not shown).

The *A. aceti* PrsAH6 construct was obtained (pJK631), however, the protein was found to be insoluble (data not shown). Co-expression with GroESL did not improve its solubility (data not shown), so the *E. coli* protein was revisited. The protocol for prep 5 was adapted from a study on the human ortholog which took advantage of PrsA

precipitating in the presence of streptomycin sulfate [8]. The purification gels are shown in Figure 3.6.

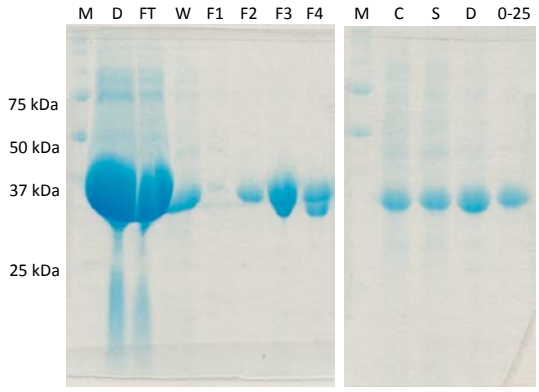

Figure 3.6 Purification of *E. coli* H6PrsA (pJK670).

Left, elution, 10 uL/lane (protein amount unknown). Right, final purification gel, 5 ug protein/lane. SDS-PAGE (12% acrylamide). Legend: D - dialyzed extract after streptomycin precipitation; FT - flowthrough; W - wash; F - fraction; 0-25 - ammonium sulfate fractionation.

A doublet in the elution fractions was observed which may indicate proteolytic products, therefore no protein was pooled off of the Ni-NTA column. However, since the column was overloaded and much recombinant protein was found in the flowthrough, an ammonium sulfate step (0-25%) was performed. A total of 95 mgs was obtained with a specific activity of 3.7 U/mg. Future isolations of *E. coli* H6PrsA should omit the Ni-NTA column. After dialysis of the resuspension of the streptomycin pellet, a simple 0-25% ammonium sulfate cut should yield pure H6PrsA.

E. coli GlnA. GlnA, or glutamine synthase, catalyzes the ATP-dependent condensation of glutamate with ammonium to yield glutamine.

E. coli H6GlnA Y397F (pJK642). pJK668 [1] contained multiple base insertion at the N-terminus, a silent G157 mutation, and a Y397F mutation, which is one of the sites of adenylation [10]. The Y397F mutant was reported to have a lower turnover number but is less subject to feedback inhibition and monovalent cation inhibition [10] therefore pJK668 was used as a PCR template to yield pJK642. For prep 1, a very low yield of

protein was obtained by affinity chromatography (data not shown) despite reprocessing the flowthrough. Prep 1 enzyme activity was not tested. A Zn precipitation protocol was attempted as previously reported and described [9, 10], however, it did not work for H6GlnA as most of the expressed protein was insoluble (data not shown). The insolubility of H6GlnA was not observed for prep 1. The N-term His-tag may interfere with the homomultimeric complex formation which led to the failure of zinc precipitation therefore the untagged protein construct was obtained.

E. coli GlnA (pJK650) purification. The zinc crystallization protocol was used to isolate untagged GlnA as shown in Figure 3.7.

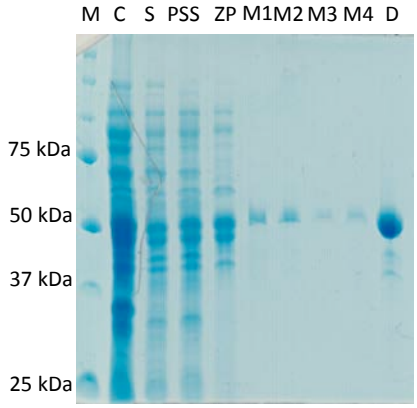

Figure 3.7 Purification of EcGlnA (pJK650).

Legend: C, crude; S, soluble; PSS, post streptomycin sulfate; ZP, Zn precipitation pellet; M1-M4, magnesium steps 1-4; D, dialyzed protein. SDS-PAGE (9% acrylamide), 10 uL (protein amount unknown) except for the last lane which contained 5 ug protein.

10 mg of protein were isolated, and despite the low yield, the Zn precipitation protocol yielded pure material. Activity was not used to track the protein therefore the appropriate resuspension time was unknown (for this prep, the pellets were resuspended for 1 hour). The specific activity of purified GlnA for this prep was 3 U/mg compared to 50 U/mg as previously reported using the Zn crystallization method [9].

E. coli H6PurL (pJK556). Large PurL from *E. coli* will be used as a control for FS activity. The purification gels are shown in Figure 3.8.

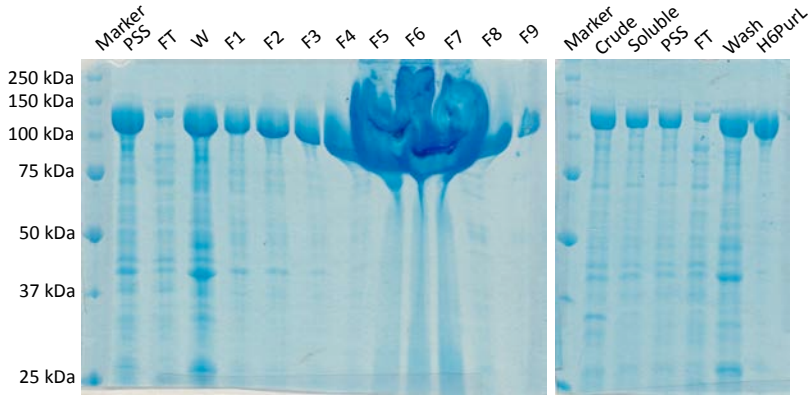

Figure 3.8 Purification of EcH6PurL (pJK556).

Left, elution profile, 10 uL/lane (protein amount unknown). Right, final gel, 5 ug protein/lane. Legend: PSS, post streptomycin sulfate; FT, flowthrough; W, wash; F, fraction. SDS-PAGE (9% acrylamide).

H6PurL was found to be a very soluble protein. A total of 136 mgs were isolated; however activity was not tested.

E. coli H6PurM (pJK633). PurM catalyzes the cyclization of FGAM to aminoimidazole ribonucleotide (AIR). FGAM, the FS substrate, is unstable, but AIR, the product of the PurM reaction, which follows FS in the de novo pathway, is not. Additionally, the Bratton Marshall assay is very sensitive to diazotizable amines, including the exocyclic one on AIR. Sequencing of the pJK675 [1] revealed a G324E mutation. Site-directed mutagenesis was performed to yield pJK633 and the protein was isolated as shown in Figure 3.9.

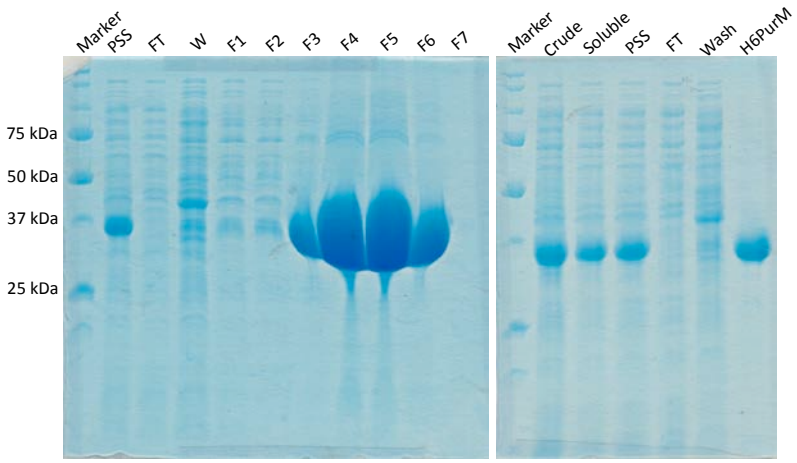

Figure 3.9 Purification of H6PurM (pJK633).

Left, elution gel, 10 uL/lane (protein amount unknown). Right, final purification gel, 5 ug protein/lane. SDS-PAGE (12% acrylamide). Legend: PSS- post streptomycin sulfate; FT - flowthrough; W - wash; F - fraction.

75 mgs of protein was isolated in this prep. Activity has not yet been tested.

3.4 Conclusion

All the constructs for the enzymes needed for FGAR biosynthesis have been obtained. *E. coli* PurF (pJK649), H6PurD (pJK621), H6PlsA (pJK669), and H6PrsA (pJK670) have been purified and shown to be active. Activity was not determined for *E. coli* H6PurL and H6PurM since the other enzymes needed for FGAR synthesis were not available at the time. PurTH6 (pJK515) was tested more than one year post-purification and low activity was observed. C and N-term His-tagged PurT have not been characterized; however one study has demonstrated that MBP-PurT was active [16] so H6PurT or the untagged protein may be a better candidates than PurTH6. Purification of GlnA (pJK650) still needs to be optimized. Although the zinc precipitation protocol seemed to yield a significant amount a material, the amount of time required to fully resuspend the various pellets after zinc and magnesium precipitation to fully recuperate active GlnA still has to be determined (*i.e.* by monitoring activity). Once GlnA is

obtained, combinations of the purine (PurF, PurD, and PurT) and regeneration pathway enzymes (PrsA, PlsA, and GlnA) will be able to be mixed together in one reaction to synthesize FGAR as proposed in Figure 3.1.

3.5 References

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CHAPTER 4. THE AAFS OPERON

4.1 Introduction

Previous observations in the AaFS protein co-expressions and functional complementation profiles (Chapters 1 and 2, respectively) have determined that functional AaFS does not contain OrfY and that the presence of the latter affects PurQ expression. If OrfY does have a regulatory role for AaFS, its position allows for the encoding of the much smaller PurS protein without expending too much energy on transcribing and translating PurQ and PurL (Figure 4.1).

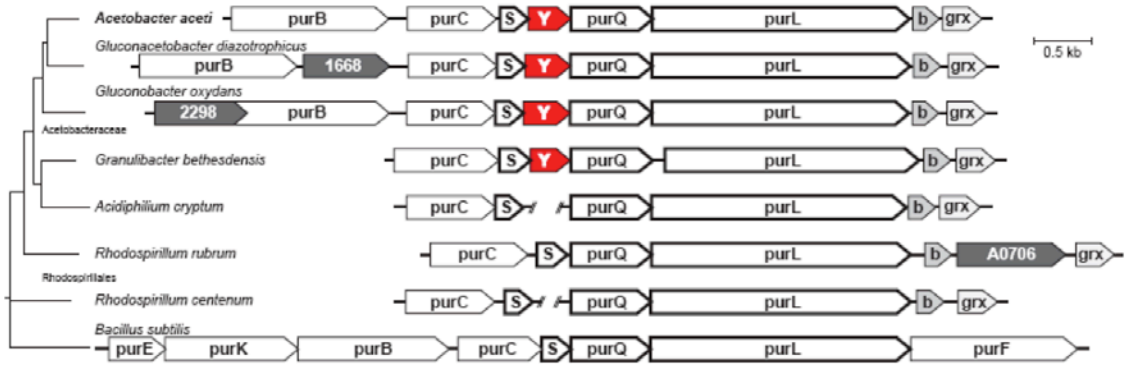

Figure 4.1 FS gene cluster alignment in select Gram-negative α-proteobacteria. Alignment includes *A. aceti*, Gram-negative *E. coli*, and Gram-positive *B. subtilis*. To date, acetic acid bacteria are the only Gram-negative bacteria that may contain PurS, PurQ, PurL, and even OrfY in some. Legend: S = *purS* ; Y = *orfY*.

As mentioned previously, *purS*, *orfY*, *purQ*, and *purL* all overlap (by four nucleotides) and are likely co-translated. The current hypothesis is that *purC*, *purS*, *orfY*, *purQ*, *purL*, *bolA*, and *grx* are transcribed on the same transcript. *purC* is 28 bp upstream of *purS*, *purL* 26 bp upstream of *bolA*, which is 26 bp upstream of *grx*. *purB* is located 155 bp upstream of *purC* and it is unclear if *purB* is co-transcribed/translated with the

remaining genes. In *B. subtilis*, all the *de novo* purine genes are clustered [1, 2]; however this is not the case in *A. aceti*.

Since OrfY shows a repressive effect on PurQ expression by SDS-PAGE (Chapter 1), OrfY may have a regulatory role. Previously reported purine pathway regulation mechanisms include feedback inhibition, riboswitches, and the presence of a transcription repressor protein [3-5]. Previous studies elucidated the negative feedback inhibition mechanism of purines on the first enzyme of the *de novo* purine biosynthetic pathway, PurF [6, 7], as well as the presence of a repressor protein, PurR, that binds the promoter region and prevents transcription from occuring [8]. Another method of regulation is at the mRNA level, where upon the presence of purines, RNA forms a secondary structure, preventing the ribosome from binding [2].

A prediction of the secondary structure of OrfY mRNA by RibEx [9] reveals the potential formation of an attenuator sequence within the *orfY* gene (Figure 2).

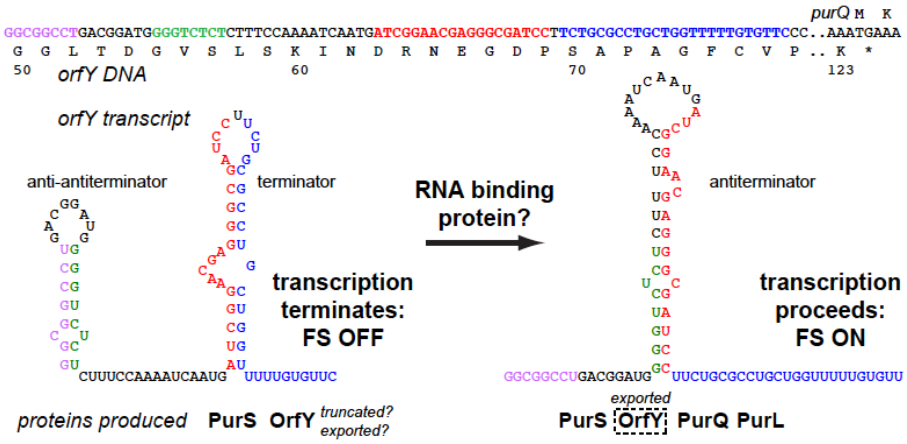

Figure 4.2 RibEx prediction of the attenuator formation within *orfY*.

Top, DNA coding sequence and corresponding amino acids of OrfY. Bottom, the formation of an anti-antiterminator loop allows for the formation of a terminator loop thus terminating transcription and turning off FS activity. Upon binding of an unknown RNA binding protein, the anti-antiterminator loop cannot form but instead an antiterminator loop which allows for transcription to proceed and produce an active FS.

The hypothesis presented in Figure 4.2 follows that of the *B. subtilis* pyrimidine pathway [10]. In short, in *B. subtilis,* the RNA binding protein PyrR interacts with mRNA carrying pyrimidine synthesis genes in the presence of uridine nucleotides, causing changes in the mRNA secondary structure and inhibiting transcription. Similarly, in the regulation of AaFS, the absence of a currently unknown orfY mRNA binding protein would allow for the formation of an anti-antiterminator loop and therefore a terminator loop leading to the termination of transcription of AaFS. Upon the binding of the RNA binding protein to the orfY mRNA sequence, an antiterminator loop is able to form which allows for transcription to proceed and produce an active FS. The export of OrfY may or may not affect the attenuator hypothesis. This chapter focuses on the isolation and characterization of RNA from *A. aceti*, specifically the AaFS operon.

4.2 Materials and Methods

Materials

All materials and chemicals were from Sigma Aldrich or Fisher Scientific and of the highest purity unless otherwise noted. Taq polymerase was from the Chapple laboratory (Purdue University) or Promega. SuperScript II and Mu-MLV reverse transcriptases were from Invitrogen and NEB, respectively.

Table 4.1 Oligodeoxynucleotides used in this chapter.

ODN	Sequence (5'=>3')[a]
404 (aka 404AC)	GAAACATGGCGCGCATTGCAGACGGCCTCT
410	GATATACATATGACCTCCTTACATTCTCC
614	CCCACCCATATGATGAGCGAAACCGCCC
622	GATCATCATACTTGCCGC
1012*	CATACCCGGCAGATcTGCAGCTCCAC
2130	GAGACAGGACATATGCTGCGAGATC
2299	CCAGTTGGTTGATTTCAAACTGGAG
2300	AAAGCGATGATGTGGTCTTCTGAAA
2301	GCGTGGGTAAGGTGATTGAACTG
2302	GGCCTTTTTCTGGGCTTCCTC
2303	ATGGGGTCTCTCTTTCCAAAATCAA
2304	GAGCGCATAACGGGCATGGT
2305	CATCAACAGCATTGCGGGTATTCT
2306	GAAGCCCTGTTTCCGTTAAAATCTG
2307	CGCTATCTGGCTTGTGTAGCAAAC
2308	CCTGATAGGGTTCGATAAAGGATGG
2309	GGGATGGTGATCATTATGCCTGTT
2310	CTGCCGGATATAGGTTTCGAGTTCT
2311	TTTCCTTGAACAGGTTTTGCAGTTC
2312	GGCGCGTATTCATTGGTAGT
2313	AATTCTGCGCCGATAATGTC
2322	AAACTAGTCCATATTTGGCCCGGTTAGC
2323	CCATCGATTGGATCCTGCAGAAATTC
2326	GCTATAAGAAGGGTGCGCTTCA
2327	TGTTTTACAGTCCTGTGCTGATCTG
2370	TTGAAGCCGAACTGGTGCG
2371	CCACGCTCCTACGTAACG

*Primer originally designed for mutagenesis

Table 4.2 Plasmids used in this chapter.

pJK	Vector	Description	Source
537	pDrive	*AamutS*	This study

Aa denotes *A. aceti* gene.

Construction of *A. aceti mutS* construct plasmid pJK637. A 1.5 kb product was amplified from *A. aceti 1023* genomic DNA, using Taq Polymerase from the Chapple laboratory (Purdue University) and ODNs 2322 and 2323 [11] with an annealing temperature of

55°C and melting, annealing, and extension times of 20 s, 30 s, and 1 min 30 s for 25 cycles. The product was purified using a Qiagen PCR purification kit and ligated into pDrive with the Qiagen QIAexpress UA cloning kit following the manufacturer's instructions. The plasmid encodes for a fragment of *A. aceti* MutS.

PCR controls. Previously isolated *A. aceti* 1023 genomic DNA (E. A. Mullins) was used as the template for PCR using ODNs from Table 2 and Taq polymerase (Chapple laboratory, Purdue University) with an annealing temperature of 55°C and melting, annealing, and extension times of 30 s, 30 s, and 30 s for 25 cycles.

Denaturing formaldehyde agarose gel electrophoresis. A 1% gel was used to check RNA integrity. A 50 mL gel was prepared by melting 0.5 g of agarose in 36 mL of water. 5 mL 10X MOPS buffer (0.4 M MOPS pH 7.0, 0.1 M NaOAc, 0.01 M EDTA), 1.8 mL 37% formaldehyde (0.22M final), and 5 uL 1 mg/mL ethidium bromide was added once the melted agarose had cooled slightly. Once solidified, the gel was presoaked for 30 min in 1X MOPS buffer and prerun for 10 min at 70 V.

RNA quantification. The Nanodrop 2000 (Thermo Scientific, Inc.) was used. 1.25 uL of sample was analyzed after blanking the instrument in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). For later O.D. readings, samples were diluted from 1:20 to 1:50 for quantification. Typically, 3 reads were averaged.

RNA isolation. Buffers including lysozyme, proteinase K, and TE were made in-house in RNase-free water (autoclaved MilliQ water in RNase-free setting); all other buffers were provided by the Qiagen or Ambion kits.

RNA isolation using Qiagen RNeasy Mini Kit. *A. aceti* 1023 cell pellets grown in YPD for 36-38 hrs (OD600 ~1.1) were stored at -80˚C and treated with Qiagen RNAProtect according to the manufacturer's instructions. In short, 1 volume of *A. aceti* culture was incubated with 2 volumes of RNAProtect reagent for 5 min, after which cells were pelleted and stored at -80°C for up to 4 weeks until use. Cell pellets were thawed on ice. 15 mg/mL lysozyme was added to 1 mL TE buffer (30 mM TrisHCl, 1 mM EDTA, pH

8.0), and 20 mg/mL proteinase K was added to a separate 1 mL TE buffer. 200 uL of lysozyme-TE buffer and 20 uL of proteinase K-TE buffer solutions were added to the cell pellets and resuspended by pipetting. The mixture was vortexed for 10 s and incubated at room temperature for 30 min. Samples were vortexed for 10 s every 2 min. 700 uL of Buffer RLT (Qiagen) was added to each sample and mixed by vortexing. 10 uL of BME was added for every mL of buffer RLT. 500 uL of 100% EtOH was added to each sample and mixed by pipetting up and down. Lysate was transferred to the spin columns in 700 uL aliquots. Columns were centrifuged for 30 s at 8,000g. Liquid was discarded and the process was repeated until all the lysate was processed. 350 uL of RW1 (Qiagen) was applied to the spin column and centrifuged. 10 uL of Qiagen DNase I stock was added to 70 uL Buffer RDD (Qiagen) and 80 uL of the DNase-RDD solution was added to the column membrane. The columns were incubated at room temperature for 15 min. 350 uL of RW1 buffer was added to the column, incubated for 5 min, and removed by centrifugation. The on-column DNase treatment was repeated once more. The column was washed twice with 500 uL RPE buffer (Qiagen). The column was placed in a new 1.5 mL tube, and eluted with 50 uL RNase-free water by centrifuging for 1 min. The same 50 uL was used again to elute more RNA from the same column. An additional in-solution DNase treatment was performed where the reaction was incubated at 37°C for 30 min. The RNA was aliquoted, frozen, and stored at -80C until use. Alternatively, New England Biolabs DNase was used in the same way as Qiagen's.

RNA isolation using Ambion PureLink RNA Mini Kit. *A. aceti* 1023 cell pellets grown in YPD for 36-38 hrs (OD600 ~1.1) were stored at -80˚C. *E. coli* DH5α cells were grown to saturation overnight and the cells were used immediately. The cell pellet (*E. coli* or *A. aceti*) was thawed on ice. Following the manufacturer's instructions, 100 uL of lysozyme solution (1 mg lysozyme in 10 mM Tris-HCl pH 8.0, 0.1 M EDTA) was added to the cell pellet with resuspension by vortexing. 0.5 uL of 10% SDS (w/v) solution was added and mixed by vortexing. 350 uL of lysis buffer (with 3.5 uL βME) was added and mixed by vortexing. For the homogenization of the cells, the lysate was passed 5 times through an 18-gauge needle with a syringe. The homogenized lysate was centrifuged at 12,000g for 2 min at room temperature. The supernatant was transferred to a clean

RNase-free tube. 250 uL of 100% ethanol was added to the cell homogenate and mixed by vortexing to disperse any precipitate. The sample (including any precipitate) was transferred to a Spin Cartridge with collection tube. After a spin at 12,000g for 15 s at room temperature, the flowthrough was discarded. 350 uL of Wash buffer I (Ambion) was added to the column and spun at 12,000g for 15 s. On-column DNase digest was performed here; otherwise, 500 uL of Wash Buffer II (Ambion) was added to the column and spun. After 1 min spin to remove any residual solvent, RNA was eluted by placing the spin cartridge into a clean RNase-free recovery tube. RNase-free water (Ambion) was added to the column and incubated at room temperature for 1 min. The RNA was eluted by centrifuging at 12,000g for 2 min. The elution step was repeated once more.

On-column NEB DNase treatment. 50 uL of the DNase mixture (5 uL 10X reaction buffer; 1.25 uL 200 mM EDTA, 1 uL DNase, 42.75 uL RNase-free water) was loaded onto the column. The column was incubated at 37˚C for 10 min. 350 uL of Wash buffer I was added and spun and the rest of the protocol above was followed.

On-column Qiagen DNase treatment. As described previously, 10 uL of Qiagen DNase I stock was added to 70 uL Buffer RDD and 80 uL of the DNase-RDD solution was added to the column membrane. The columns were incubated at room temperature for 15 min. 350 uL of Wash buffer I was added and spun and the rest of the protocol above was followed.

A. aceti RNA isolation using hot acid phenol. Cells from 10 mL of culture were harvested and the supernatant was discarded. Cell pellets were frozen for storage at -80˚C or used immediately. Cells were resuspended in 400 uL freshly made and cold AE buffer (50 mM NaOAc pH 5.2, 10 mM EDTA pH 8.0) and transferred to an RNase-free tube. 40 uL of 10% SDS (w/v) and 400 uL acid phenol:chloroform (Amresco Phenol:Chloroform::5:1) was added. The mixture was vortexed briefly and placed in a 65˚C heat block for 10 min, vortexing for 5 s every minute. Cells were incubated on ice for 5 min and spun for 5 min at 14,000 g at 4˚C. The aqueous (upper) layer was transferred to a new tube and 300 uL chloroform was added. The tube was gently shaken

to mix and spun for 5 min at 14,000 g at 4˚C. The aqueous layer was transferred to another tube. 50 uL of ice cold 3M NaOAc (pH 5.2) and 1 mL ice cold 100% ethanol was added to the tube and mixed by inverting several times. The tube was chilled at -80˚C for 30 min or at -20˚C overnight and spun down for 10 min at 14,000 g at 4˚C. Ethanol was removed, the pellet was rinsed with ice cold 70% ethanol, and the tube was spun for 5 min at 14,000 g at 4˚C. As much of the 70% ethanol was removed using a pipettor and the pellet was allowed to air dry (~5 min at room temperature). The pellet was resuspended in 100 uL of RNase-free water by vortexing and samples were stored at -20°C or -80°C.

In solution Qiagen DNase treatment. 87.5 uL DNA-contaminated RNA (at ~1 ug/uL [RNA]), 10 uL RDD buffer, and 2.5 uL DNaseI reconstituted DNase I were mixed together. The reaction was incubated at 37˚C for 30 min. DNase was removed by phenol:chloroform extraction as described above.

In solution cDNA synthesis. Invitrogen SuperScript II or NEB M-MuLV Reverse Transcriptase (RT) was used according to the manufacturers' instructions. In short, 100-500 ng total RNA, 2 pmol of gene specific antisense primer, and 0.5 mM dNTPs were mixed in a tube in a final volume of 12 uL. The RNA mixture was heated to 65°C for 5 min and chilled on ice. 1X Buffer, 10 mM DTT, and 200 U of enzyme was added to the RNA mixture and the whole reaction was incubated at 42°C for 30 min. The reverse transcriptase was heat inactivated at 65°C for 20 min.

Two-step RT-PCR. 1 uL of the cDNA reaction above with 200ng RNA input was used for the +RT reactions. 200ng of total RNA was used for the -RT reactions. GoTaq polymerase (Promega) or Taq polymerase from the Chapple laboratory (Purdue University) was used for the amplification reactions. 30 cycles were run.

rRNA depletion. The Ambion MICROBExpress™ Bacterial mRNA Enrichment Kit was used according to the manufacturer's instructions. In short, 10 ug RNA (subjected to 2 in-solution Qiagen DNase treatments) in a maximum volume of 15 uL was added to 200 µL Binding Buffer in a 1.5 mL tube and the tube gently vortexed to mix. 4 µL of

Capture Oligo Mix was added to the RNA in Binding Buffer and the tube was gently vortexed to mix. The tube was microfuged briefly to get the mixture to the bottom of the tube. The RNA/Capture Oligo reaction was heated to 65°C for 10 min and incubated at 37°C for 15 min. During this incubation, the Oligo MagBeads were prepared by resuspending the beads by vortexing. 50 uL of beads were aliquoted into a 1.5 mL tube. The beads were captured using a magnetic stir bar and the supernatant was removed carefully and discarded. The beads were washed by adding 50 uL of nuclease-free water and resuspending the beads by brief, gentle vortexing. The beads were recaptured and the supernatant was removed and discarded. The beads were equilibrated by adding 50 uL of Binding Buffer and resuspending them by brief, gentle vortexing. The beads were recaptured and the supernatant was removed and discarded. The Oligo MagBeads were resuspended in 50 uL Binding Buffer, and the slurry was incubated at 37°C until use. The Wash solution was heated to 37°C. The washed and equilibrated beads were resuspended by gentle vortexing and 50 uL was added to the RNA/Capture Oligo Mix. The reaction was gently vortexed to mix, microfuged briefly, and incubated for 15 min at 37°C. The beads were recaptured and the supernatant (containing the enriched mRNA) was carefully aspirated into a Collection Tube on ice. 100 uL of preheated Wash Solution was added to the Oligo MagBeads and the latter were resuspended by brief, gentle vortexing. The beads were recaptured and the supernatant was pooled with the RNA already in the Collection Tube on ice. The enriched mRNA was ethanol precipitated by adding 1/10th volume of 3 M sodium acetate, 1/50th volume 5 mg/mL glycogen, and 3 volumes ice cold 100% ethanol. After mixing thoroughly by vortexing, the RNA was precipitated at –20°C for 1 hr. The tube was centrifuged for 10 min at 14,000g and the supernatant was decanted and discarded. 750 uL of ice cold 70% ethanol was added to the RNA pellet and the tube was vortexed briefly. The tube was centrifuged for 5 min at 14,000g. The supernatant was discarded and an additional wash was performed. Any remaining supernatant was removed with a pipettor and the pellet was air dried for 5 min. The RNA pellet was resuspended in 10 uL TE (10 mM Tris-HCl pH 8, 1 mM EDTA) by vortexing. Any remaining Oligo MagBeads were removed here

by capturing the beads and removing the enriched mRNA solution to a new RNase-free tube. Samples were stored at -20°C or -80°C.

4.3 Results and Discussion

PCR controls. *A. aceti* 1023 genomic DNA was used to test primers designed for RT-PCR. Figure 4.3 shows the general annealing region for the AaFS cluster.

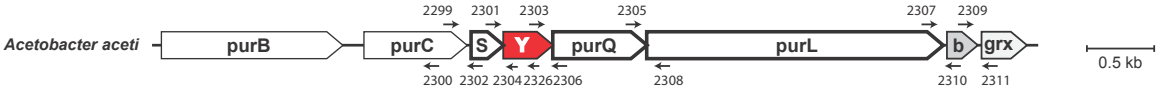

Figure 4.3 ODN priming sites for the AaFS operon.

Primers were designed to yield amplification products ~750 bp as summarized in Table 4.3 for all amplicons used in this chapter. PCR controls for the AaFS and *purXEK* operons are shown in Figure 4.4 and 4.5, respectively.

Table 4.3 RT-PCR amplicon characteristics summary.

Spanning	**Expected Size (bp)**	**ODN (fwd)**	**ODN (rev)**
purC-purS	458	2299	2302
purS-orfY (post-attenuator)	393	2301	2304
purS-orfY (pre-attenuator)	153	2301	2326
orfY-purQ	500	2303	2306
purS-purQ	790	2301	2306
purQ-purL	454	2305	2308
purL-bolA	254	2307	2310
bolA-grx	485	2309	2311
purF (short)	167	2312	2313
purF (long)	397	2371	2313
mutS (short)	200	2327	2322
mutS (medium)	662	2370	2322
mutS (long)	1363	2323	2322
orfX-purE	541	2130	1012
purE-purK (short)	246	404	622
purE-purK (long)	744	614	622

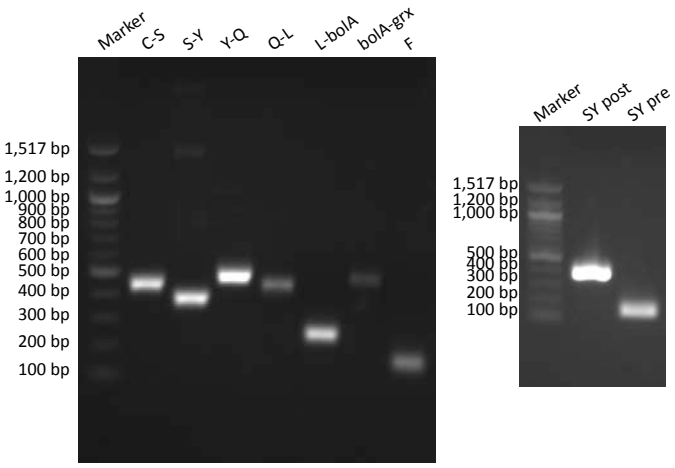

Figure 4.4 PCR controls for the AaFS operon of *A. aceti* 1023.
5 uL/lane, 1.4% agarose.

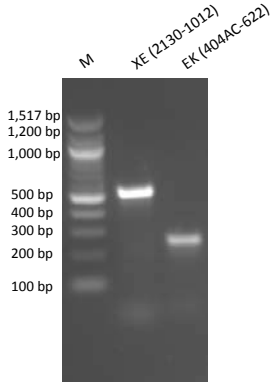

Figure 4.5 PCR controls for the *purXEK* operon of *A. aceti* 1023.
5 uL/lane, 2% agarose.

The primers designed for RT-PCR were shown to work on *A. aceti* 1023 genomic DNA so RNA isolation from *A. aceti* was the next step.

RNA isolation optimization. Very low yields of total RNA (<40 ng) were obtained when the Qiagen or the Ambion kits were used. Note that buffers including lysozyme, proteinase K, and TE were made in-house in RNase-free water; all other buffers were provided by the Qiagen or Ambion kits. Kit-isolated samples could not be visualized by denaturing formaldehyde agarose gels and RT-PCR results showed extensive genomic DNA contamination (data not shown). Elizabeth Tran suggested acid phenol extraction

since cell pellets would not be used for any other assays (*i.e.* kinetic characterization) and to increase isolated RNA yield. Additionally, in-solution DNase treatments appeared to be more effective than on-column treatments (data not shown). The final preps included two in-solution DNase I treatments.

MutS, involved in DNA double-strand break repair, was previously established as a qRT-PCR control for *Acetobacter pasteurianus*, as growth under various stressors such as heat, higher ethanol concentrations, and acidic conditions in the growth media did not affect *mutS* transcription levels [11]. Since *A. aceti* does not have a characterized housekeeping gene and is closely related to *A. pasteurianus*, MutS was chosen as a control. Additionally, *purF* was chosen as a purine pathway control since PurF catalyzes the first committed step in *de novo* purine biosynthesis [12].

A successful total RNA isolation with no detectable genomic DNA contamination by PCR is shown in Figure 4.6.

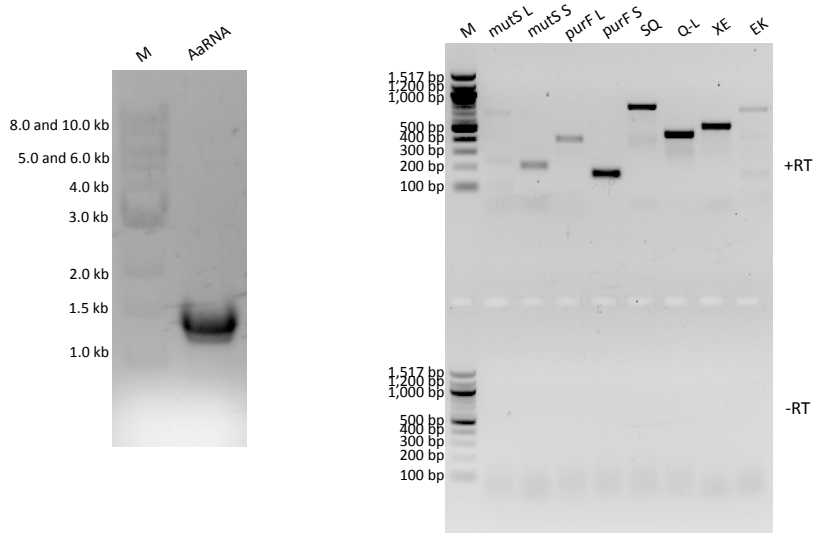

Figure 4.6 Total RNA from *A. aceti* 1023.

Left, denaturing formaldehyde agarose gel (1%, 5 ug RNA). Right, RT-PCR, 2% agarose, 5 uL/lane (200 ng starting RNA). 30 cycles of PCR. 2 amplicon sizes were tested for *mutS* and *purF* (S- short; L- long). M - marker. NB - the "mutS L" product here corresponds to the "mutS medium" product in Table 4.3.

A. aceti ribosomal RNA, unlike *E. coli* rRNA, appears to have two distinct bands at ~1.2 kb. 10 mL of culture would typically yield ~100 ug of total RNA. Two in-solution DNase treatments followed by 30 cycles of RT-PCR showed a strong signal in the +RT reactions, and no amplification from contaminating DNA in the -RT reactions. Smaller amplicons, except for *mutS* and *purF*, were found to amplify contaminating genomic DNA even after three DNase treatments (data not shown), therefore larger amplicons were used for characterization of the AaFS and *orfX-purE-purKK* operons. The transcripts for *orfY* and *orfX* seem to be encoded on the same mRNA as AaFS and PurE-PurK, respectively.

Transcriptome analysis via RNA-Seq requires enrichment of mRNAs (as recommended by Phillip San Miguel from the Purdue University Genomics Core Facility). 10 ug of the total RNA isolated in Figure 4.6 was used as the input for rRNA depletion by the Ambion MICROBExpress Bacterial mRNA Purification kit, which relies upon hybridization of capture oligonucleotides to rRNA. RT-PCR results post-rRNA depletion are shown in Figure 4.7.

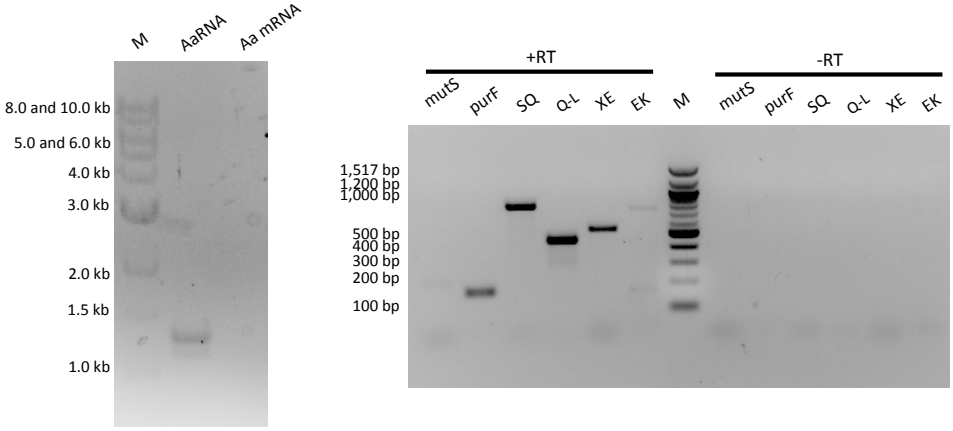

Figure 4.7 Enriched mRNA from *A. aceti* 1023 and RT-PCR.

Left, denaturing formaldehyde agarose gel (1%) before (AaRNA, 1ug) and after rRNA depletion (Aa mRNA, 50 ng). Right, RT-PCR, 2% agarose, 5 uL/lane (200 ng starting RNA). 30 cycles of PCR. M - 100 bp marker.

No detectable genomic DNA contamination was observed by RT-PCR, even after mRNA enrichment (Figure 4.7). Very faint amplification products were observed in the *mutS* and *EK* lanes, which was not the case for *purF*, *SQ*, *QL*, and *XE*. Ambion mentioned that the typical yield of enriched mRNA would be roughly 10% of the input total RNA (therefore from the recommended 10 ug total RNA, 1 ug of mRNA should be obtained). The yield of mRNA was ~30 ng. The absence of the 23S and 16S bands by denaturing agarose gel electrophoresis or the Agilent Bioanalyzer is the only indicator of (presumably) successful rRNA depletion. The latter method has been implemented as a typical quality control step for next generation sequencing (*i.e.* RNA-Seq).

A. aceti total RNA and rRNA-depleted mRNA samples were sent for a quality control check on the Agilent Bioanalyzer. Allison Sorg from the Purdue Genomics Center noted that the RNA concentrations (by Nanodrop) were very low. They recommend submitting 2 ug of RNA with an RNA integrity number (RIN) of 8 of higher before proceeding forward to making a library and sequencing. On a scale of 1-10, a high RIN indicates a high quality RNA sample [13]. Bioanalyzer traces for total RNA and rRNA-depleted samples can be accessed through the following link: http://www.genomics.purdue.edu/cgi-bin/cgiwrap/core/wiki.cgi/hr_00571_kappock_acetobacter_1_2013-06-05. The RIN for total RNA and rRNA-depleted *A. aceti* 1023 samples were determined to be 7.1 and 8.6, respectively. It is important to note that in order to get a RIN number for the rRNA-depleted sample (AA2), Allison Sorg changed the threshold of zero due to the low concentration of the RNA. Peaks at ~1200 and 1400 nt are rRNA peaks, which were also observed by denaturing formaldehyde agarose gels in lanes containing total RNA (Figures 4.6 and 4.7). The peak at 25 nt is a standard. For the total RNA sample, peaks at ~100 nt may be degradation products. A strong signal was observed for the rRNA peaks, which decreased significantly to below 15 FU after rRNA-depletion, indicating that mRNA enrichment with the MICROBExpress kit is compatible with *A. aceti* RNA. The Life Technologies website lists compatible, partially compatible, and incompatible bacteria for the kit, where *A. aceti* was not listed. Although the rRNA depletion was not complete, a significant amount of ribosomal RNA was removed from total RNA despite

the low yield. More mRNA is required for RNA-Seq therefore one suggestion is to process multiple rRNA-depletion reactions, pool, and concentrate the enriched mRNA in order to proceed forward.

orfY was established to be on the same mRNA transcript as *purSQL*, therefore the other genes (*purC*, *bolA*, and *grx*) in the gene cluster were also tested as shown in Figure 4.8. ODN 2311 was used for cDNA synthesis of the whole gene cluster (starting from grx) using the same batch of mRNA-enriched sample that was sent to the BioAnalyzer.

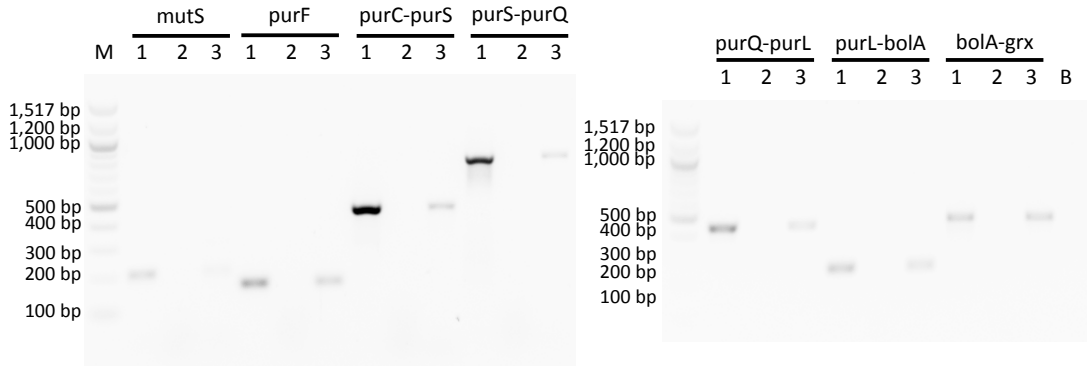

Figure 4.8 RT-PCR for the AaFS gene cluster

2% agarose, 5 uL/lane from 200 ng starting RNA. 30 cycles of PCR. Legend: M - 100 bp marker; 1 - *A. aceti* genomic DNA template (PCR control), 2 - *A. aceti* total RNA template (-RT); 3 - *A. aceti* cDNA template (+RT); B - blank lane.

No contaminating genomic DNA was observed in the -RT reactions. Amplification products were observed for all targets in the +RT reactions, therefore *bolA* and *grx* are likely transribed on the same mRNA as *orfY* (Figure 4.8).

4.4 Conclusion

RNA was successfully isolated from *A. aceti* 1023 grown in YPDE via the hot acid phenol extraction method. When total RNA is analyzed by denaturing formaldehyde agarose gel electrophoresis, two rRNA bands were observed at ~ 1.2 kb. mRNAs for

mutS, *purF*, *orfX-purE-purK*, and *purC-purS-orfY-purQ-purL-bolA-grx* were detected by RT-PCR. *orfX-purE-purK* and *purC-purS-orfY-purQ-purL-bolA-grx* were found to be on the same mRNA transcript, respectively. For mRNA enrichment, the Ambion MICROBExpress kit was found to be compatible with *A. aceti* 1023 although a scale-up will be required to obtain enough material for quality control on the Agilent Bioanalyzer (and ultimately RNA-Seq).

In Chapters 1 and 2, OrfY was shown to have a regulatory role on PurQ and potentially PurL protein expression and in functional complementation studies. However, due to its proximity to *bolA* and *grx* in the gene cluster (Figure 4.1), *bolA* and *grx* being found on the same mRNA transcript (Figure 4.8), and four conserved cysteine residues in the OrfY amino acid sequence (Chapter 1), OrfY may have a role in iron-sulfur cluster biogenesis. None of the genes in the operon encode for a Fe-S cluster-containing protein... but there is an iron-sulfur protein in the *de novo* purine biosynthetic pathway, PurF. PurF in *B. subtilis* [14] and chicken [15] were shown to contain a Fe-S cluster; *E. coli* does not. *A. aceti* PurF is predicted to also contain an Fe-S cluster (T. J. Kappock, unpublished observations). Now that *bolA* and *grx* were shown to be on the same mRNA transcript as *orfY*, the current hypothesis is that in purine-starvation conditions, PurF, OrfY, BolA, and Grx (and PurL) would all be expressed together. Preliminary work on developing a defined minimal medium for *A. aceti* 1023 to turn on (or off) purine genes can be found in Chapter 5.

4.5 References

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CHAPTER 5. *A. ACETI* 1023 GROWTH IN MINIMAL MEDIA

5.1 Introduction

A. aceti has been used for the commercial production of vinegar for centuries [1]. Multiple cytoplasmic *A. aceti* proteins have been characterized [2-5] and shown to be acid, and coincidentally, thermo-stable [2-5].

Propagation of *A. aceti* in rich medium simply uses YPDE [2]; however a defined minimal medium has not been developed for culturing *A. aceti* 1023.

5.2 Materials and Methods

All materials and chemicals were from Sigma Aldrich or Fisher Scientific and of the highest purity unless otherwise noted. CSM-Ade-His-Leu-Trp was from BIO 101, Inc. Yeast nitrogen base without amino acids was from Difco. Strains used in this chapter are summarized in Table 5.1. MilliQ water was used for all media prep.

Table 5.1 Strains used in this study.

Organism	Strain	Genotype
Acetobacter aceti	1023	wild type
Saccharomyces cerevisiae	W1588-4C	*MAT*a *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*

YPDE. 10g yeast extract, 20g bacto-peptone, and 20g dextrose [6] were sterilized by autoclaving. 2% (v/v) ethanol was supplemented prior to inoculation when YPD was cooled down. A single colony of *A. aceti* 1023 was used to inoculate 500 mL of YPDE in a 2.8 L baffled Fernbach flask and grown at 30°C (200 rpm).

Minimal media. Media were supplemented with hypoxanthine (1.5 ug/mL) wherever applicable. All cultures contained thiamin (10 uM final) unless otherwise indicated.

Ohmori medium. As previously described [7, 8]. Detailed composition is summarized in Table 5.2. Cells grown to $O.D._{600}$ ~1 were washed with 1/15 M Na, KP_i, pH 6.5 buffer and used to inoculate 100 mL of Ohmori medium in 500 mL Klett meter flasks. Cultures with starting $O.D._{600}$ ~0.005-0.1 were grown at 30°C (200 rpm). "Modified Ohmori" medium was Ohmori medium supplemented with the AMM metals. "Ohmori+" medium was modified Ohmori supplemented with 2% (w/v) peptone.

Table 5.2 Summary of minimal media composition.

	pH 5.0	pH 6.5	pH 6.0
	Acetobacter Minimal Medium	**Ohmori**	**modified Ohmori**
K_2HPO_4		0.57 mM	0.57 mM
KH_2PO_4	7.3 mM	3.67 mM	3.67 mM
$(NH_4)_2SO_4$	25 mM		25 mM
$MgSO_4(7H_2O)$	1 mM	1 mM	1 mM
$CaCl_2(2H_2O)$	0.1 mM	0.68 mM	0.68 mM
$Na_2MoO_4(2H_2O)$	0.001 mM		0.001 mM
$FeCl_3(6H_2O)$		0.03 mM	0.03 mM
$CuSO_4$	2E-4 mM		2E-4 mM
$MnCl_2$	0.006 mM		0.006 mM
$ZnSO_4$	0.006 mM		0.006 mM
$FeSO_4(7H_2O)$	0.013 mM		
Na Glutamate		23.7 mM	23.7 mM
KCl		1.3 mM	1.3 mM
Glucose	2%	3%	3%
Reference	[9]	[7]	

Complete supplement medium (CSM). Plates were prepared as described [10]. Plates were kept at 30°C for 72 hrs.

Supplements

Amino Acid mix. A 1:1000 stock was used to supplement minimal media. The final concentrations of 1.5 nM each of Asp, His, Ile, Leu, Phe, Val, Ala, Lys, and Arg; 1 nM Trp and Tyr were used.

UHWL mix. A 1:1000 stock was used to supplement minimal media. The final concentrations of 1.5 ug/mL uracil, 1 mM His, Leu, Trp were used.

Vitamin mix. A 1:1000 stock was used to supplement minimal media. The final concentrations of 0.1 nM biotin and 1 nM pantothenate were used.

5.3 Results and Discussion

YPDE propagation. *A. aceti* 1023 was shown to exhibit biphasic growth [11, 12] in rich medium. *A. aceti* propagation in YPDE was monitored up to the first log phase to determine when cells should be harvested for future growths in various minimal media (Figure 5.1).

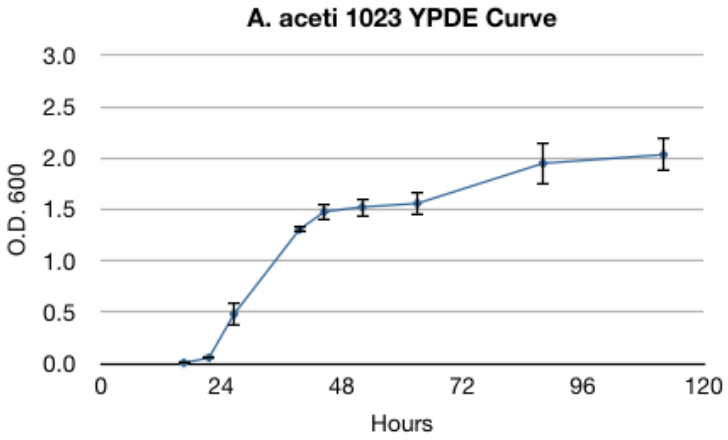

Figure 5.1 Propagation of *A. aceti* 1023 in YPDE.

The growth curve for *A. aceti* 1023 was similar to that of previous studies [11, 12]. The first log phase was determined to end ~36-40 hrs post-inoculation with an $O.D._{600}$~0.75-1.

A. aceti minimal media propagation.

Multiple attempts at growing *A. aceti* 1023 on Ohmori medium [7] yielded unreproducible and unpredictable results. Some cultures grew after 72 hrs as previously observed [8] while others did not grow even after 10 days (data not shown). When

supplemented with 0.2% peptone however, cultures in both Ohmori and AMM media showed some growth, even though $O.D._{600}$ did not surpass 0.4 (data not shown).

Aeration was important as cultures grown in baffled flasks reached a higher O.D. Ohmori medium supplemented with peptone in a baffled flask, followed by plain Ohmori medium in a baffled flask, seemed to work well for *A. aceti* growth. AMM, which was designed for *Acetobacter xylinus* [9], was not as good of a medium for *A. aceti*, therefore it will not be used for subsequent studies. Ohmori *et. al* did not mention the addition of micronutrients [7]; however sterilized tap water, which would contain metals, may have been used. Tap or deionized water have not been tested for *A. aceti* growth using the Ohmori medium salts.

Propagation of *A. aceti* in the modified Ohmori medium (Ohmori + AMM micronutrients) is shown in Figure 5.2.

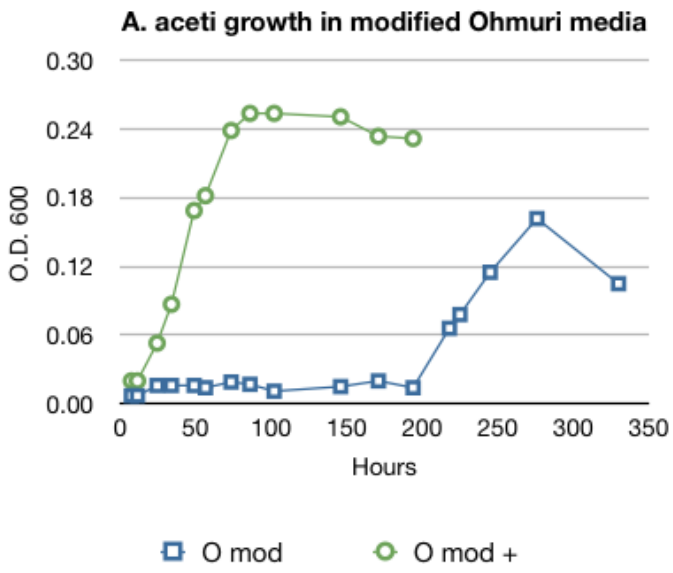

Figure 5.3 *A. aceti* 1023 propagation in supplemented Ohmori medium. Supplementation with AMM micronutrients (O mod, ☐) or with AMM micronutrients and 0.2% peptone (O mod +, ○).

Cultures with or without peptone in Figure 5.3 were inoculated at a lower starting $O.D._{600}$, 0.005 rather than 0.1, since there was ambiguity about the complete depletion of nutrients and if cultures had reached the end of the log phase. Growth was observed in the presence of peptone where the stationary phase was reached in ~ 100 hrs, compared to > 200 hrs for the media lacking peptone. Peptone may contain some nutrient that *A. aceti* requires for growth in minimal medium.

Thiamin dependence of *A. aceti*. The *de novo* purine and thiamin biosynthetic pathway share the same first five steps. The necessity of thiamin supplementation in minimal medium for *A. aceti* was unknown and always added to minimal media. The thiamin dependence of *A. aceti* is shown in Figure 5.3.

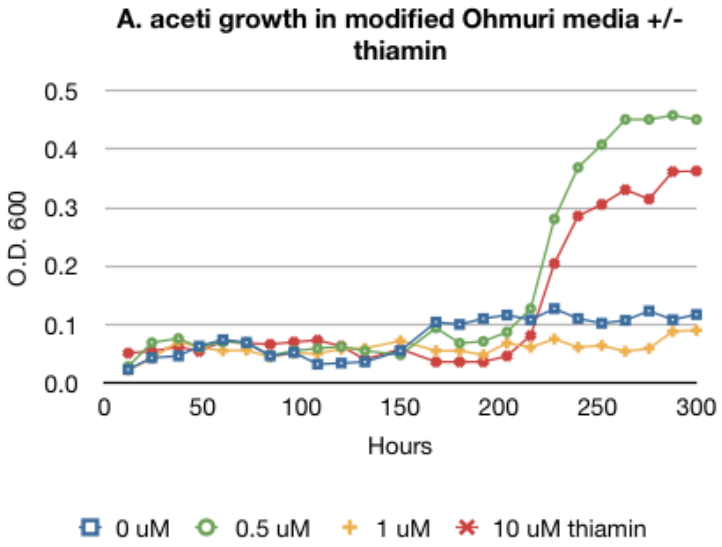

Figure 5.3 Thiamin requirement of *A. aceti* in modified Ohmori medium.
NB - addition of thiamin may have been omitted in the 1 uM curve.

Previous studies in the lab utilized 10 uM thiamin, but it seems that 0.5 uM is sufficient, although the culture containing 1 uM thiamin grew the most poorly (addition of thiamin in that flask might have been omitted). Thiamin seems to be required in order for *A. aceti* to grow since the culture without any thiamin grew to $O.D._{600}$ ~0.1. No peptone was added in this experiment, where *A. aceti* grew very slowly, reaching stationary phase

after 10 days. *A. aceti* may need some other nutritional requirement that is in peptone. An amino acid mix designed from the results of a JGI/DOE search for *A. aceti* amino acid requirements is listed in the Methods and Materials and was tested below with supplementation with the vitamin mix.

S. cerevisiae was used as a positive control to ensure that the media tested could sustain growth since *A. aceti* did not yield reliable results thus far. *S. cerevisiae* W1588-4C is a purine auxotroph and contains the *ade2* (PurE-PurK fusion) mutation. *ade2* colonies appear red due to the accumulation and conversion of aminoimidazole ribonucleotide (AIR) to the red compound. W1588-4C, when streaked on YPD, had the expected red pigmentation. Growth of pink colonies was observed on modified Ohmori plates supplemented with peptone (data not shown) and those results were also seen in liquid media (Figure 5.4).

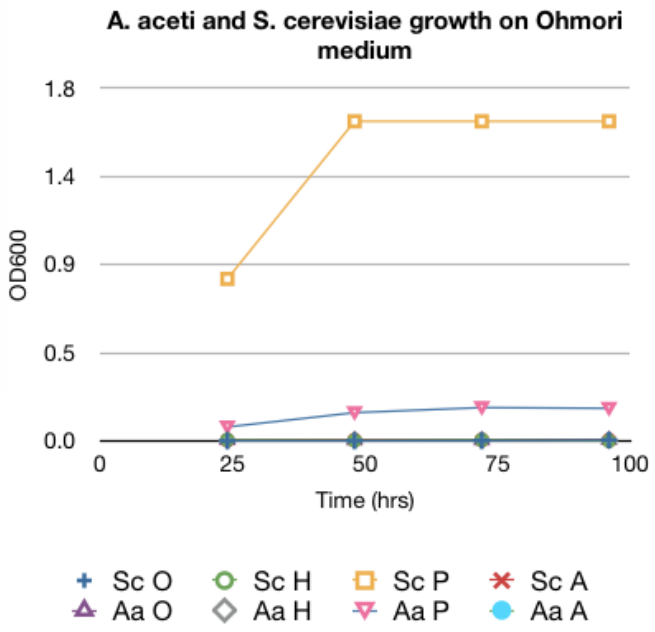

Figure 5.4 Growth curves of *A. aceti* and *S. cerevisiae* on modified Ohmori medium. Legend: Sc, *S. cerevisiae*; Aa, *A. aceti*; O, Ohmori unsupplemented; H, +hypoxanthine; P, + peptone; A, +amino acid/vitamin mix.

In liquid medium, *S. cerevisiae* grew well in the presence of peptone, and although *A. aceti* did not grow as well, there was some observable growth in the peptone-supplemented medium. These results indicate that the use of peptone will not yield purine starvation conditions since *S. cerevisiae* W1588-4C, a purine auxotroph, grew, indicating that peptone contains purines (and potentially pyrimidines). No growth was observed for either organism in modified Ohmori medium, modified Ohmori medium supplemented with hypoxanthine, or modified Ohmori medium supplemented with the amino acid/vitamin mixes.

Additional modified Ohmori plates were poured where a mix containing uracil, tryptophan, histidine, leucine, and adenine were included to ensure growth of *S. cerevisiae* as a positive control. Hypoxanthine was also tested since it had been used in the past as the go-to purine; however *A. aceti*'s preference of hypoxanthine over adenine (or vice versa) was unknown.

Table 5.3 Ohmori Plate results.

	S. cerevisiae	*A. aceti*
Ohmori (water)	-	+
Ohmori (peptone)	+++	+++
Ohmori (UWLH)	+	+
Ohmori (UWLH ade)	+++	+++
Ohmori (UWLH hyp)	+++	++
Ohmori (aa)	+++	+++
Ohmori (aa ade ura)	+++	+++
Ohmori (aa hyp ura)	+++	++

Legend:
+++ growth observed after 96 hrs
++ growth observed after 144 hrs
+ growth observed after 2 weeks
- no growth after 2 weeks

Although colonies were very small, a lawn was observed. The amino acid/vitamin and the uracil/trp/leu/his mixes seem to also have contaminating purines as *S. cerevisiae* was able to grow, albeit not well, on both. Although the dry amino acid stocks may have been contaminated with purines (obtained from old labs), another possibility is the conversion of AIR to carboxyaminoimidazole ribonucleotide (CAIR) in the presence of carbon

dioxide. A denser lawn of *A. aceti* was observed on the Ohmori+peptone plate when compared to the Ohmori+amino acid/vitamin mix and the UWLH mix plates. Additionally, *A. aceti* seems to prefer adenine as a source of purines over hypoxanthine as growth was observed on adenine 2 days before that on hypoxanthine-supplemented plates. Another option to try next is yeast nitrogen base (YNB), used in complete synthetic medium (CSM) or synthetic complete (SC) medium, for *S. cerevisiae*. Yeast nitrogen base contains defined salts, vitamins, and micronutrients; SC medium supplements YNB with amino acids, adenine, and/or uracil, and a carbon source. No growth was observed for either *A. aceti* or *S. cerevisiae* at the first attempt using solid CSM medium, possibly due to too large of a dilution when plating cells and the short amount of time (72 hrs) the plates were left at 30°C. CSM and SC differ in the amount of supplied amino acids, where CSM contains less of them [10].

5.4 Conclusion

Growth of *A. aceti* in Ohmori medium could not be reproducibly observed, even when micronutrients were added (modified Ohmori). Some growth was observed when peptone was present, but an unknown key nutrient maybe found in tap water (not yet tested) is required by *A. aceti*. *S. cerevisiae* was used as a positive control in order to ensure that any manipulations would not yield toxic effects. *A. aceti* was found to require proper aeration (baffled flasks), thiamin supplementation, and a preference for adenine over hypoxanthine. Further work needs to be done in finding a defined minimal medium for *A. aceti* 1023 as peptone contains purines and has the disadvantage of batch-to-batch variability. CSM and SC media, which both utilize YNB, have been developed as defined minimal media for yeast. Since *A. aceti* and *S. cerevisiae* have similar culturing conditions (growth at 30°C; propagation in YPD for yeast, YPDE for the acidophile), CSM and SC could be explored as potential culture media for *A. aceti* even though growth was not observed during the first attempt on solid CSM medium.

5.5 References

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CHAPTER 6. MISCELLANEOUS WORK

6.1 Materials and Methods

All materials and chemicals were from Sigma Aldrich or Fisher Scientific and of the highest purity unless otherwise noted. Oligodeoxynucleotides (ODNs, Table 1) were obtained from IDT. OneTaq and Vent polymerases were obtained from NEB. FlexiGoTaq polymerase was purchased from Promega. Chapple Taq polymerase was isolated in the Chapple laboratory by Joann Cusumano (Purdue University).

Table 6.1 Oligodeoxynucleotides used in this chapter.

ODN	Sequence (5'=>3')[a]
603/850/2124	TAATACGACTCACTATAGGG
851/2125	GCTAGTTATTGCTCAGCGG
2138	GGATCTCGACGCTCTCCCT
2139	ATGCGTCCGGCGTAGA
2140	TTGTACACGGCCGCATAATC
2141	GATTATGCGGCCGTGTACAA
2283	TTCCTTAAGCTTCTAAGACTAAACCGTGGCTTTTGCAATAC
2284	TTCCTTGAATTCTTAGCCGATTTTGTTACGTTGTGCG

Table 6.2 Plasmids used in this chapter.

pJK	Vector	Description	Source
358	pET23a	*uctD*	[3]
551	pET28a	*H6uctD*	This study
634	pDrive	*evgA* R153W	This study

Construction of *H6uctD* construct plasmid pJK551. pJK358 was digested with NdeI and EcoRI and ligated into the NdeI and EcoRI restriction sites of the destination vector pET28a to yield pJK551. The plasmid encodes for *A. aceti* H6UctD.

Construction of *evgA* R153W construct plasmid pJK634. A Taq PCR product obtained by K. Nyffeler using ODNs 2283 and 2284 was purified using a Qiagen PCR purification kit and ligated into pDrive with the Qiagen QIAexpress UA cloning kit following the manufacturer's instructions. The plasmid contains the endogenous promoter region of EvgA and encodes for *A. aceti* EvgA R153W.

Colony PCR protocol. Protocol adapted from [1]. In short, PCR reactions were set up as delineated in Tables 6.3, 6.5 and 6.7 and amplified according to the conditions in Table 6.4, 6.6, and 6.8, respectively. Colonies were first picked from transformation plates, patched onto a fresh LB-antibiotic plate, and the remainder of the colony on the pipette tip was transferred into the PCR reaction by pipetting up and down 5-6 times while avoiding the introduction of air bubbles. Occasionally, to obtain more material, colonies were patched on fresh LB-antibiotic plates first and left to grow for 4-6 hrs at 37°C prior to colony PCR. Antibiotics were ampicillin at 100 ug/mL, streptomycin at 50 ug/mL, or kanamycin at 70 ug/ml final concentrations. PCR products were analyzed on 0.7%-1.5% agarose depending on the size of the amplification products. Controls usually consisted of a PCR reaction using colonies carrying the parent vector (pET23a, pET23d, pET28a, pET-Duet, or pCDF-Duet) or the actual plasmid as template.

Table 6.3 Cocktail for Vent.

Vent polymerase		
Mix	**[Final]**	**Volume (uL)**
10X ThermoPol buffer	1X	3
MgSO4	5 mM	1.5
Primer 1 (5 uM)	0.25 uM	1.5
Primer 2 (5 uM)	0.25 uM	1.5
dNTPs (2.5 mM)	200 uM	2.4
Template		
Vent	0.3 U	0.3
Water		19.5
	total:	30 uL

Table 6.4 PCR conditions for Vent.

Conditions	**Vent**		
Step	**Temp (C)**	**Time**	
Hot start	98	5 min	
Init. Den.	98	30 sec	
Den.	98	20 sec	repeat 25X
Anneal	55 (varies!)	20 sec	
Extension	72	1 min/kb	
Final Ext.	72	7 min	
Hold	4	infinity	

Table 6.5 Cocktail for OneTaq.

OneTaq polymerase		
Mix	**[Final]**	**Volume (uL)**
5X OneTaq Buffer	1X	6
Primer 1 (5 uM)	0.25 uM	1.5
Primer 2 (5 uM)	0.25 uM	1.5
dNTPs (2.5 mM)	200 uM	2.4
Template		
OneTaq		0.5
Water		18.1
	total:	30 uL

Table 6.6 PCR conditions for OneTaq.

Conditions	**OneTaq**		
Step	**Temp (C)**	**Time**	
Hot start	94	5 min	
Init. Den.	94	30 sec	
Den.	94	20 sec	repeat 25X
Anneal	55 (varies!)	20 sec	
Extension	68	1 min/kb	
Final Ext.	68	7 min	
Hold	4	infinity	

Table 6.7 Cocktail for Taq.

Taq (Chapple or Promega)		
Mix	**[Final]**	**Volume (uL)**
5X Flexi GoTaq Buffer	1X	6
Primer 1 (5 uM)	5 uM	1.5
Primer 2 (5 uM)	5 uM	1.5
MgCl2 (25 mM)	1.25 mM	1.5
dNTPs (2.5 mM)	200 uM	2.4
Template		
OneTaq		0.5
Chapple Taq (1:50 dil)		1.5
Water		18.1
	total:	30 uL

Table 6.8 PCR conditions for Taq.

Conditions	**Taq**		
Step	**Temp (C)**	**Time**	
Hot start	95	5 min	
Init. Den.	95	30 sec	
Den.	95	20 sec	repeat 25X
Anneal	55 (varies!)	20 sec	
Extension	72	1 min/kb	
Final Ext.	72	7 min	
Hold	4	infinity	

ESI-MS of *A. aceti* proteins. OrfYH6 from prep 1 (Chapter 1) and previously isolated *A. aceti* proteins were analyzed on a Thermo Scientific LTQ (Parker laboratory) with S. Ouellette's assistance. Samples were diluted in 50:50:01 water: acetonitrile: formic acid. A minimum of the top 3 peaks from each MS spectrum was input into ESIprot1.0 [2] for analysis.

6.2 Results and Discussion

Colony PCR. Figure 6.1 shows the annealing sites for the ODNs (listed in Table 6.1) used for screening construct candidates. It is important to note that gene specific primers can also be used in this protocol.

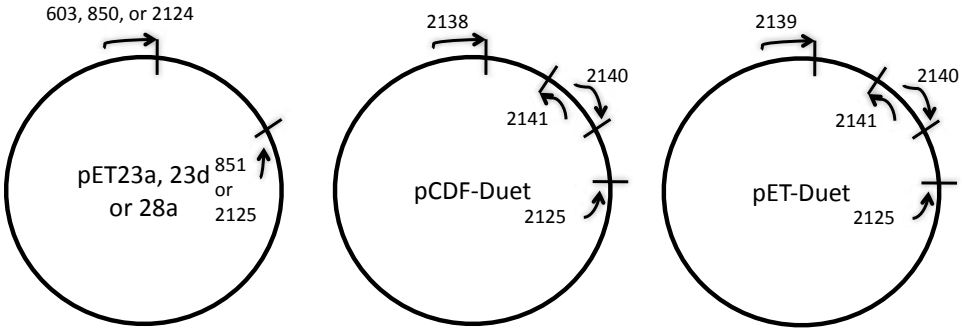

Figure 6.1 Colony PCR annealing sites.
Plasmids not to scale.

Colony PCR was used as a method to screen for candidates as an alternative to restriction mapping, the latter which requires isolating the plasmid first and digesting the DNA prior to running a gel. If colony PCR failed, restriction mapping was used to screen constructs. Use of the empty vector as a control ensured that the PCR reaction worked and determined a baseline where successful ligation of inserts would yield an amplification product larger than ~250 bp (size of the T7 promoter-terminator region).

Cloning. H6UctD (pJK551) was obtained for comparison with the untagged protein (Benjamin Carter and Funmi Adebesin rotations). EvgA R153W (pJK634) was obtained for UctB and acid adaptation of *A. aceti* studies (Kayleigh Nyffeler).

ESI-MS. Steven Ouellette (Parker Lab) ran some samples on their LTQ. A minimum of the top 3 peaks from each spectrum were analyzed by ESIprot 1.0 [2] and the deconvoluted data obtained is summarized in Table 6.9.

Table 6.9 ESI-MS results as analyzed by ESIprot 1.0 [2].

Sample	**Dilution**	**Expected Mass (Da)**	**Calculated Mass (Da)**	**Std Dev.**	**Difference (Da)**	**% Diff.**
PurE H59Q*	1:100	18856.8	18859.95	1.77	-3.15	-0.02
E. coli PurE (500)	1:100	17741.94	17677.03	19.24	64.91	0.37
E. coli PurE (501)	1:100	17741.94	17697.95	37.28	43.99	0.25
507*	1:50	18788.37	18788.99	1.66	-0.62	0
508*	1:100	18765.34	18765.2	0.44	0.14	0
509*	1:100	18804.37	18801.85	0.92	2.52	0.01
510*	1:100	18804.37	18804.72	1.11	-0.35	0
526*	1:20	18804.37	18809.82	18.62	-5.45	-0.03
527*	1:100	18804.37	18809.82	1.53	-5.45	-0.03

**A. aceti* genes

6.3 References

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